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THE EFFECT OF AN ANABOLIC STEROID AND TRAINING ON THE SKELETAL MUSCLE OF THE HORSE

A THESIS

PRESENTED FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

BY

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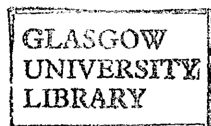

TO THE HORSES

J.W., DIRK, ADAM, ASTRYL,

OSCAR, SMOKEY and BEN

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This thesis is based in part on the following papers:

1) Influence of fixation on the ultrastructure of horse skeletal muscle following exercise. Nimmo, M.A. and Snow, D.H. Proceedings Royal Microscopical Society 12, 57 (1977).

2) The effects of an anabolic steroid on equine skeletal muscle. Nimmo, M.A., Snow, D.H. and Munro, C.D. IV International Congress on the Biochemistry of Exercise, 1979 (in press).

3) Effects of the anabolic steroid nandrolone, in the resting horse. Snow, D.H., Munro, C.D. and Nimmo, M.A. Equine Vet. Journal, 1980 (in press).

SUMMARY

1) Resting Study

Nandrolone phenylpropionate (therapeutic dose) was administered i.m. to 4 healthy, mature castrate horses at weekly intervals for 7 weeks. Measurement of body weight and nitrogen excretion failed to show a consistent anabolic effect. Examination of biopsies from the semitendinosus and biceps femoris muscles showed no alteration in water content or glycogen concentration with treatment. Similarly no changes were found in the activities of the following enzymes: lactate dehydrogenase, aldolase, citrate synthase, aspartate aminotransferase, alanine aminotransferase and 3-hydroxyacyl CoA dehydrogenase. However a significant increase in the proportion of fast twitch low oxidative fibres of the biceps femoris was observed. The occurrence of some components of stallion behaviour in geldings was taken to reflect a residual androgenic activity in the compound.

2) Exercise Study

The effect of nandrolone phenylpropionate was investigated in 6 Thoroughbred geldings which were involved in a training programme. The study was conducted on a cross-over basis, each animal acting as its own control.

(a) The steroid did not affect body weight or body measurements. The initial stimulus of training, however, caused decreases in weight and in flank measurement. There was no consistent anabolic effect noted in the nitrogen

excretion values and they were not affected by training.

(b) Biopsies were obtained from the biceps femoris semitendinosus and middle gluteal. Water content of the samples was not altered by training or training/anabolic steroid administration.

(c) Glycogen concentration increased in control and anabolic groups of all muscles after training except the anabolic group of the biceps femoris.

(d) The activities of enzymes, representing the glycolytic pathway, lactate dehydrogenase and phosphofructokinase, were not affected by training or training/anabolic steroid administration. Citrate synthase and 3-hydroxyacyl CoA dehydrogenase increased significantly with training in the anabolic group but although they increased in the control group the increase was not significant. However cytochrome oxidase increased significantly in both the anabolic and control group. β-Glucuronidase did not alter significantly with respect to training or training/anabolic steroid administration.

(e) Histochemical differentiation of fibre types was carried out on the middle gluteal and biceps femoris. Training caused an increase in the percentage fast twitch high oxidative fibres in the anabolic and control group of the middle gluteal and the control group of the biceps femoris. The percentage fast twitch high oxidative fibres in the anabolic group of the biceps femoris after training was not significantly different from pretraining values.

(f) Fibre area analysis on histochemical sections

stained for succinate dehydrogenase showed no significant difference between control and anabolic group in either the biceps femoris or middle gluteal, nor were the areas affected by training. Ratios of high oxidative:low oxidative fibre areas did not alter with training or training/anabolic steroid administration in the middle gluteal or with training in the biceps femoris. The high oxidative:low oxidative ratio of the anabolic group of the biceps femoris increased significantly with training. In all muscles there was an increase in area occupied by the high oxidative fibres.

(g) Capillary number increased with training in both muscles. There was no difference between control and anabolic group. However the capillary number per unit fibre area did not increase with training in either group of either muscle.

3) Acute Exercise Study

(a) Electron microscopy study. The effect of strength and endurance exercise on skeletal muscle ultrastructure was investigated in 3 Thoroughbreds and 1 Heavy Hunter using various fixation procedures. The resting ultrastructure was similar to that of other species. After strength exercise disruption was noted in all samples. Disruption was also noted after endurance exercise but only in samples fixed in osmium tetroxide.

(b) Metabolites. The 6 Thoroughbred horses of the exercise study were galloped over various distances 506m, 1025m, 1600m and 3620m. Glycogen depletion was significantly greater in the 1025m and 1600m than in the 506m trial.

Pyruvate concentrations did not alter significantly with exercise. Lactate production over the 3 shorter distances was significantly greater than the production over the 3620m trial. There was no difference at any stage, in any parameter measured, between control and anabolic group.

In conclusion, these studies have shown that at a high therapeutic dose, nandrolone phenylpropionate had little effect on the skeletal musculature of both resting and exercising Thoroughbred horses. The only notable effect was the alteration in histochemical profile of the biceps femoris muscle. The relevance of this change to the racing performance of the whole animal is questionable. Both the electron microscopical study and the investigation into the utilisation of fuels over various distances provides some preliminary results which serve as a good basis on which to continue.

ABBREVIATIONS

The following abbreviations were used in this thesis:

CP	creatine phosphate
ATP	adenosine triphosphate
ADP	adenosine diphosphate
Pi	phosphate
CHO	carbohydrate
FFA	free fatty acids
ST	slow twitch
FT	fast twitch
FTH	fast twitch high oxidative
MAD	methylandrostenediol
TCA	tricarboxylic acid
Vo_2 max	maximum oxygen uptake
HAD	3-hydroxyacyl CoA dehydrogenase
CS	citrate synthase
LDH	lactate dehydrogenase
PFK	phosphofructokinase
Cyt. ox.	cytochrome oxidase
β gluc	β-glucuronidase
AST	aspartate amino transferase
ALT	alanine amino transferase
ALD	aldolase
E/M	electron microscope
HO	high oxidative
LO	low oxidative
BF	biceps femoris
MG	middle gluteal
Semi-T	semitendinosus

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SECTION 1

INTRODUCTION AND LITERATURE SURVEY

INTRODUCTION

To learn about movement is very largely to learn about Nature, for Nature has been defined as "the principle of motion and change"(Aristotle: Oxford Translation, 1831).

Vertebrates move along the ground in a variety of ways. Some of the finest early reports are by P. G. Newcastle (1657) on the "Gaits of Horses" and Giovanni Borelli's "De Motu Animalium" (1679). Since that time the rich diversity of vertebrate locomotion has received a great deal of attention and one of the main features common to all is the generating force, skeletal muscle. The muscles make up about the same fraction of the total body mass in all mammals, that is around 45% irrespective of the body size (Munro, 1969). Skeletal muscle function during locomotion can be divided into at least three types. There are isotonic contractions used in swinging the limbs forward and also in tensioning the tendons when the animal is running at speed. There are isometric or near isometric contractions involved in tensing the tendons so that they store the maximum amount of energy before impact and, just after impact there are also some negative work contractions as the muscles are stretched whilst they are still generating force. This is particularly true when an animal is running down a slope.

Because of the various functions which muscle

has to perform it is not surprising to find that the muscles of animals are adapted specifically to suit their function and that even within a particular muscle the fibres are not homogeneous. The ratios of the numbers of the different types of fibre vary with animal size (Davies and Gunn, 1971).

That there is some specificity in the response and adaptation of muscle to different types of exercise is apparent. Champion athletes in different sports vary considerably. When male and female distance athletes are compared to controls they can be characterised by a relatively high proportion of oxidative fibres (Prince, Hikida and Hagerman, 1976) which are related to the ability to perform low intensity work for a prolonged time (Hultman, 1967) whereas shotputters can be characterised by relatively larger fibre areas (Thorstensson, 1976) which is related to strength (Ikai and Fukunaga, 1968). These differences are attributable both to heredity (Komi, Viitasalo, Havu, Thorstensson and Karlsson, 1976) and to the specific type of training used by the athlete (McCafferty and Horvarth, 1977).

In world sport, over the past 20 years, the need to "win" has grown all important and with it has grown the study of exercise physiology and the development of new training methods. Although training is a major component in producing a maximum performance, it is inevitable that trainers and athletes will seek an easier alternative. The administration of anabolic

steroids is one of the most commonly used short-cuts despite their world-wide ban in human sport. In horse-racing they are banned only in Europe; in America and Australia anabolic steroids are frequently used on the race-track.

The justification for assuming an improved performance with anabolic steroids is equivocal. The reports claiming an improved performance with anabolic steroids have often not had adequate controls and there are many which do not show any improved performance, only the production of undesirable side effects (Ryan, 1976). Because of the controversial information on anabolic steroids when used in conjunction with a training programme, it was decided that a well-controlled trial should be carried out to look at the effects of an anabolic steroid on healthy mature animals. The study was planned in such a way that it was also possible to look at the effects of a particular training régime on skeletal muscle and in particular those aspects which can be investigated by using histochemical and biochemical techniques. The animals selected were Thoroughbred horses as they represent the élite of their species in that they have been bred for their ability to perform well over short distances.

LITERATURE SURVEY

HISTOCHEMISTRY

It has been known for some time that mammalian skeletal muscle can be divided into "red" and "white" (Lorenzini, 1678, cited Paukul, 1904). Kühne (1865) showed that the colour difference was representative of the muscle fibre and not of the blood it contained. In 1873 Ranvier published the first results correlating the colour of the muscle with speed of contraction. His experiments on rabbits showed that "red" muscles contracted more slowly than "white" and that they were more resistant to fatigue. Shortly after Ranvier's work Paukul (1904) reported on many different properties of rabbit muscles and concluded that all "slow" muscles were "red" but not all "red" muscles were slow.

The early histological studies (Knoll, 1891; Schaffer, 1893) also revealed that mammalian muscles were heterogeneous and that the "red" and "white" fibres were, in fact, the extremes of a range in colour. Ogata (1958a, b, c) classified a further type, the intermediate fibre, but the full extent of the heterogeneity has only been revealed with new histochemical techniques over the past 20 years.

Dubowitz and Pearce (1960) subdivided muscle into two types on reciprocal differences in the activities of oxidative and glycolytic enzymes. Those which stained intensely for oxidative enzymes but were low in phosphorylase activity were termed Type I and

the converse were termed Type II. Although two classes of fibres can be characterised on the basis of an oxidative stain, within each class there is a wide spectrum of activity (Gunn, 1978; Spamer and Pette, 1979).

An alternative method of dividing skeletal muscle into fibre types is based on the histochemical myofibrillar ATP-ase (myosin ATP-ase) activities (Padvkula and Herman 1955)

In 1967, Barany (1967) showed that the activity of myosin ATP-ase was directly proportional to the speed of shortening of a muscle and Guth and Samaha (1969) were able to demonstrate that high myosin ATP-ase fibres (Type II) and low myosin ATP-ase (Type I) correlate qualitatively with the biochemical measurements.

The original histochemical reaction was carried out at a pH of 9.4 as this gave a clear division of the fibres. Later studies, however, on human and rat muscle found that the myosin ATP-ase reaction was pH labile (Brooke and Kaiser, 1970). By using an acid or alkali preincubation it was possible to subdivide the Type II fibres. The acid preincubation is well defined in human where the Type IIA fibres are distinguishable at a preincubation pH greater than 4.8 while preincubation at more acidic values results in a complete loss of staining. Type IIB fibres stain for ATP-ase after preincubation above pH 4.6 but do not react when pretreated at a lower pH. The possibility of mitochondrial ATP-ase interfering with the myosin ATP-ase reaction (Guth, 1973) has been refuted at least

for the IIA and IIB fibres in that at pH 4.35 mitochondrial ATP-ase is stable whereas the IIA and IIB stain negatively (Jansson and Kaiser, 1977). The allocation these fibre types is not dependent therefore upon interference with the mitochondrial ATP-ase. The suggestion also that acid preincubation merely denatures the ATP-ase (Khan, Papadimitriou and Kakulas, 1974) and that the fibre division does not reflect a functional division has been partly offset by electrophysiological work (Garnett, O'Donovan, Stephens and Taylor, 1978). This group correlated Type IIA and Type IIB with the degree of fatiguability and found that although both fibre types were fast twitch the IIA fibres showed a low fatigue-ability compared to the IIB suggesting a metabolic and therefore functional differentiation of the fibres.

Further histochemical pH manipulation yields further Type II subdivisions, in all, 5 types have now been reported (Ingjer, 1979a) (Table 1.1). The characteristics of the IIC, IIAB and IIAC fibres are not clearly defined. Jansson and Kaiser (1977) have found a high percentage of IIC fibres in female orienteers. They resemble Type I fibres in capillary supply, muscle fibre size, oxidative enzyme activity and mitochondrial content (Essén, Jansson, Henriksson, Taylor and Satlin, 1975; Ingjer, 1979a). Also recent immunohistochemical studies (Billeter, Weber, Lutz, Howald, Eppenberger and Jenny, 1980) have shown the IIC fibres to contain both the slow myosin of the Type I fibre and the fast myosin of the Type II. It is not yet clear whether

TABLE 1.1 Muscle fibre characteristics of human skeletal muscle

Fibre type	Biochemical profile	Contraction time	Fatigu-ability	-vely stained with pre-incubation
Type I	High oxidative Low glycolytic	Slow	Low	Outside pH 3.9 to 10.4 *
Type IIA	Intermediate oxidative High glycolytic	Fast	Low	Outside pH 4.9 to 10.8 *
Type IIB	Variable oxidative (Untrained low oxidative) Intermediate glycolytic	Fast	High	Outside pH 4.5 to 10.8 *
Type IIC	High oxidative High glycolytic	-	-	Outside pH 4.9 to 10.8 Total inhib. pH $<$ 3.9 *
Type IIAB	-	-	-	Staining characteristics between Types IIA and Type IIB **
Type IIAC	-	-	-	Staining characteristics between Type IIA and Type IIC **

* Brooke and Kaiser (1970)

** Ingjer (1979)

these fibres have a functional role or whether they are acting as intermediates undergoing transformation.

The IIAB fibres (Grønnerød, Dahl and Savage, 1977) have a fibre size, capillary supply and mitochondrial content between Type IIA and Type IIB (Ingjer, 1979a) and they probably represent a transitional state between Type IIA and Type IIB. Type IIAC is present at such a low percentage in human skeletal muscle that a study of these fibres has not yet been possible. However, it would appear that the identification of a spectrum of activity already noted with the oxidative stain is also true of the myosin ATP-ase reaction.

The metabolic differentiation of the IIA and IIB fibres noted with electrophysiological work can also be demonstrated by histochemical staining of serial sections for myosin ATP-ase pH 9.4 and an oxidative enzyme. The myosin ATP-ase reaction identifies the slow twitch (ST) from the fast twitch (FT). The oxidative stain identifies the FT fibres as being either high oxidative (FTH) or low oxidative (FT).

Most authors limit references to the three functional types that is Type I, Type IIA and Type IIB or ST, FTH and FT. Although these two classifications are not directly interchangeable in that the IIBs may differ in oxidative capacity (Snow and Guy, 1980) in general, comparisons can be made (Table 1.2).

Metabolic differentiation of muscle fibres may be dictated by the blood supply to the fibre (Brown,

Cotter, Hudlická and Vrbová, 1976) or conversely the blood supply may be dictated by the metabolic characteristics of the fibre (Andersen and Henriksson, 1977). Whichever way the dependence lies, a high correlation exists between the mitochondrial content of a fibre and the capillary supply to it (Ingjer, 1979a). The anatomical arrangement of capillary to muscle was originally studied in detail by Ranvier in 1874. Subsequently Krogh (1918-1919) analysed capillary distribution in relation to the oxygen pressure head necessary for supplying the tissue. From this detailed study, he concluded that skeletal muscle capillaries are arranged with such regularity that the number of capillaries can be estimated from a transverse section. This has allowed a number of detailed studies to be carried out particularly over recent years in human muscle subjected to exercise (see section on Plasticity) (Brodal, Ingjer and Hermansen, 1977; Andersen and Henriksson, 1977; Ingjer, 1978, 1979a, b).

BIOCHEMISTRY

Skeletal muscle is unique in that it can vary its metabolic rate to a greater degree than any other tissue. In fact working skeletal muscles can increase their oxidative processes to more than 50 times the resting level (Asmussen, Christensen and Nielsen, 1939). The source of energy for muscular work was believed by Leibig (1840, cited Cori, 1975) to be muscle protein. However other lines of work eventually led to the

TABLE 1.2 Fibre nomenclature used in the literature

Authors	Species	Nomenclature				
Ogata (1958a, b, c)	Rabbit	White		Red		Intermediate
Dubowitz and Pearce (1960)	Rat, pigeon, toad, goldfish	II		II		I
Engel (1970)	Man	II		II		I
Brooke and Kaiser (1970)	Man	IIB		IIA	IIC	I
Barnard *et al* (1971)	Guineapig	FT white		FT red		ST intermediate
Peter *et al* (1972)	Guineapig	FG		FOG		SO
Burke *et al* (1973)	Cat	FF		FR		S
Lindholm and Piehl (1974)	Horse	FT		FTH		ST
Jansson (1975)	Man	IIB		IIA	IIC	I
Guy and Snow (1977)	Horse	FT		FTH		ST
Garnett *et al* (1978)	Cat	FF		FR		S
Ingjer (1979)	Man	IIB	IIAB	IIA	IIAC IIC	I

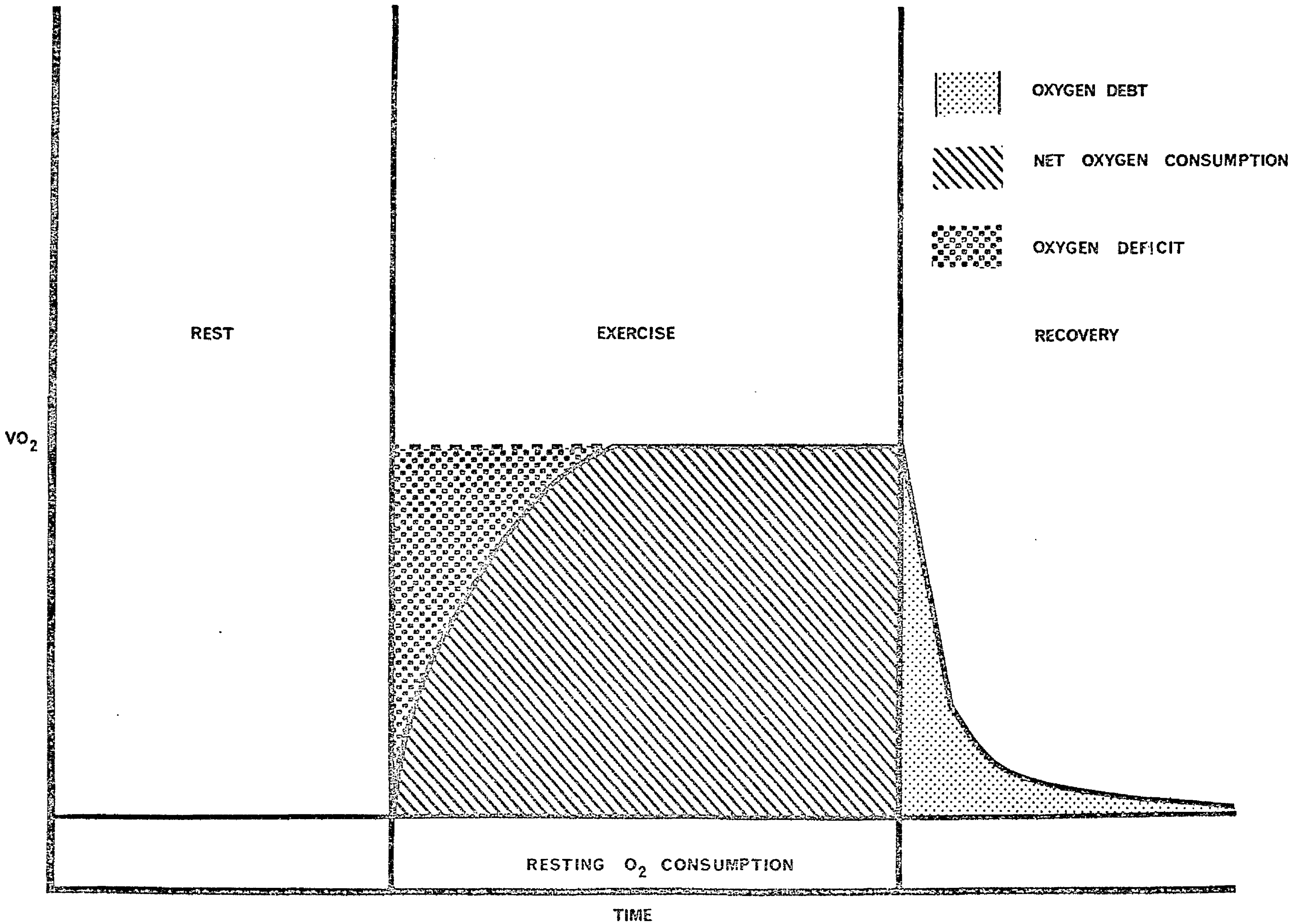
FF = Fast twitch fatiguable
FR = Fast twitch fatigue resistant
SO = Slow twitch oxidative
FG = Fast twitch glycolytic
FOG = Fast twitch oxidative/glycolytic

realisation that the anaerobic breakdown of muscle glycogen and the production of lactic acid could provide the energy for muscular contraction (Hill, Long and Lipton, 1925). This was termed the Hill-Meyerhoff theory. The intricate experiments of Hill dominated the field for several years. When muscle becomes active O_2 is required immediately but the O_2 uptake suffers a time lag. The oxygen "missed" during the initial period of adjustment is defined as the oxygen deficit. Termination of exercise is also an abrupt phenomenon followed by a time lag in the change of O_2 consumption (Figure 1.1).

Hill *et al* (1925) noticed that the recovery phase involved at least two separate processes. A "fast" component, they believed, was to support the conversion of lactate back to glycogen in the muscle and the slower part was the conversion of the circulating lactate in the liver. The period 1925-1933 saw muscle metabolism studies take a great leap forward. Creatine phosphate (CP) was discovered simultaneously by Eggleton and Eggleton (1927) and Fiske and Subbarrow (1927) and was shown to decrease during contraction and increase again during relaxation. Adenosine triphosphate (ATP) was discovered by Lohmann (1931) and the Hill-Meyerhoff theory was destroyed by Luunsgaard (1930a, b) when he showed that lactic acid was not a prerequisite for muscle contraction.

These new developments allowed Margaria, Edwards

Figure 1.1 Changes in rate of oxygen consumption during a period of rest, exercise and recovery


OXYGEN DEBT
NET OXYGEN CONSUMPTION
OXYGEN DEFICIT
REST
EXERCISE
RECOVERY
VO2
RESTING O2 CONSUMPTION
TIME

and Dill (1933) to rethink Hill's oxygen debt concept. They found that in relatively mild exercise an O_2 debt arose without any accumulation of lactate in the blood. Furthermore in severe exercise it was found that the decline in O_2 consumption did not parallel the decrease in lactate. The removal of lactate could explain the slower component of the oxygen debt. However the fast component was now attributed to the replenishment of CP and refilling O_2 stores within the muscle and was called the "alactic" debt.

Lohmann continued his work with the muscle energy stores and in 1934-1935 discovered that CP would not split into two unless ATP was in its reduced form adenosine diphosphate ADP and phosphate (Pi). This relationship of ATP, CP and ADP led to the suggestion that it was ATP which was the immediate energy source for muscle contraction.

The final link between muscle contraction and the biochemical events associated with it was made when Engelhardt and Ljubimowa (1939) discovered that the muscle protein, myosin, could split ATP to produce ADP and Pi thus releasing the necessary energy for muscle contraction. From this work and others (Kalchar, 1941; Lipmann, 1941; Krebs, 1943) the various metabolic pathways in skeletal muscles were elucidated.

Krogh and Lindhard (1920) had established that both fat and carbohydrates (CHO) could be used as substrates for the production of ATP. The proportions

to which these fuels were used was demonstrated by Christensen and Hanson (1939). It was evident that in long continued moderate exercise the relative fat utilisation increases gradually, due to the reduction in CHO stores. In exercise of increasing intensity the relative CHO utilisation increases to levels approaching 100% at maximal work. CHO and fat are available to muscle in two forms either as endogenous glycogen and triglycerides or as blood-borne glucose and free fatty acids (FFA) respectively.

The glycogen stores in muscle are so limited that if this were the only fuel, exhaustion would occur within minutes or even seconds from the initiation of muscular work. The alternative source of CHO for muscle, blood glucose, necessitates an adequate vascular system to supply glucose at a sufficient rate. In addition, the source of blood glucose, the liver, also has limited reserves. In contrast, the stores of fat in the body are large and blood-mediated FFA are the main fuel in prolonged work. It is still unclear to what degree endogenous muscle triglycerides are decreased in prolonged exercise. Carlson (1969) reached the conclusion that most of the energy demand during prolonged exercise can be met by circulating FFA. However, Essén, Hagenfeldt and Kaijser (1977) have shown triglyceride depletions in single fibre analysis of endurance runners.

The control of the metabolic pathways has received much attention over the past few years. Newsholme

and Underwood (1966) devised the theory of "substrate cycles" and their application is reviewed by Newsholme and Start (1973). The theory can be applied to fulfilling the energy demand necessary for sprinting as Newsholme (1978) has calculated that the large increases necessary cannot be afforded even by large changes in the metabolic regulators. Alternatively the large metabolic changes noted in muscle may be accounted for by the existence of a multi-enzyme complex which is in association with glycogen (Pette, 1975). Regulation may occur from differing properties of the enzymes when in the complex and when in solution. In line with this observation, Hofer (1971) had already demonstrated that muscle phosphofructokinase aggregates at high concentrations and that the dissociation-association equilibrium is influenced by the concentration of certain metabolites and effectors.

The heterogeneity of many skeletal muscles has made it difficult to obtain quantitative biochemical measurements of any one particular fibre type. However, the development of micromethods of enzyme analysis (Lowry and Passonneau, 1972) has made it possible to study metabolic characteristics of muscle tissue at the level of single fibres (Essén *et al*, 1975). Using this technique it has been found that the adaptability of the oxidative potential of skeletal muscle in humans can be found within the Type I and Type II fibres (Henriksson and Reitman, 1976). Also, the glycogen content of the

Type I and Type II fibres are comparable whereas the triglyceride concentration is almost 3 x higher in Type I as compared to Type II (Essen *et al*, 1975). An alternative method to single fibre analysis is to select muscles which are virtually homogeneous. The soleus and psoas of the rabbit are representative of slow and fast twitch respectively. Using these preparations, Spamer and Pette (1977) have been able to show that the soleus muscle shows a uniform population with regard to phosphofructokinase, lactate dehydrogenase and malate dehydrogenase, whereas the fast twitch fibres in the psoas are uniform with regard to the glycolytic enzymes but reveal pronounced variations in malate dehydrogenase. Heterogeneity was also found for 3-hydroxyacyl-CoA-dehydrogenase in psoas but the two oxidative enzymes were not proportional (Spamer and Pette, 1979).

It is not possible always to either carry out single fibre analysis or to select a homogeneous muscle but the information gained from these studies may be applied when considering a mixed fibre population.

MUSCLE PLASTICITY

The contractile and associated biochemical properties of adult skeletal muscle can be transformed from fast twitch to slow twitch and conversely slow twitch to fast twitch by changing their innervation. In this type of experiment, which was pioneered by Buller, Eccles and Eccles (1960), the motor nerves of a fast and a slow muscle were cut and cross anastomosed; as a result

the fast muscle was reinnervated by the nerve which previously supplied the slow muscle, and vice versa. The contractile characteristics then changed, the fast muscle becoming slower and the slow muscle faster. The development of a miniature electronic stimulator (Salmons, 1967) made it possible to subject a nerve, normally receiving phasic bursts of activity, to a stimulating current field which pulsates continuously at 10Hz, a typical postural pattern. The work of Salmons and Vrbová (1969), using this technique, demonstrated marked slowing in response to long term stimulation of the fast muscles of the rabbit and cat. Further work, by Salmons and Sréter (1976) demonstrated that the change was brought about by the particular pattern of activity of the motor nerve into the muscle. The sequence of events which accommodates the conversion from fast to slow muscle has now been elucidated for chronic low frequency stimulation. After only a few days of stimulation, there are functional and ultrastructural alterations of the sarcoplasmic reticulum. They are manifested by changes in ATP-ase activities and the rate and capacity of calcium transport. These initial changes are presumed responsible for the early reduction in contractile speed measured isometrically (Heilman and Pette, 1979). After a longer period of stimulation increases occur in the oxidative capacity of the fibres which are concomitant with a decrease in the glycolytic capacity. This can be demonstrated both biochemically (Pette, Müller, Leisner and Vrbová, 1976) and histochemically (Brown, Cotter, Hudlická and Vrbová, 1976). In addition,

studies monitoring the specific activity and alkali stability of the calcium-activated myosin ATP-ase activity (Salmons and Sréter, 1976) histochemical (Pette _et al_ 1976) and immuno-fluorescence (Rubinstein, Mabuchi, Pepe, Salmons, Gergely and Sréter, 1978) have demonstrated changes in both the heavy and light chains of myosin. These changes in the myosin ATP-ase activities would account for the continued reduction both in isometric contractile speed (Pette _et al_ 1976) and in the maximum speed of shortening measured under isotonic conditions (Al-Amoud, Buller and Pope, 1973). Cross-innervation studies, either fast muscle to slow or vice-versa, also lead to corresponding changes in the metabolism of the muscles, reflected by a marked re-arrangement of the enzyme activity patterns (Drahota and Gutmann, 1963) and in calcium-activated ATP-ase activity (Buller, Mommaerts and Seraydarian, 1969). However, the possibility of changing the percentage of FT and ST fibres by physiological means such as training is still under debate although there is much evidence in support of the view that the distribution of the main fibre types is largely governed by genetic factors (Komi _et al_, 1976). Metabolic properties of a muscle can, however, be altered by physical training. The literature classifies training under two main headings, endurance and strength. The former may be defined as

low tension-high repetition and the latter as high tension-low repetition activities. The first experiments on rats exposed to endurance training found no biochemical changes associated with the exercise (Hearn and Waino, 1956; Gould and Rawlinson, 1959). Later studies (Holloszy, 1967), however, found that if the exercise was sufficiently strenuous, it was possible to identify an increase in the respiratory capacity of skeletal muscle with endurance training.

It was possible to look more closely at these effects by using the percutaneous needle biopsy. This technique revived by Bergström (1962) allowed serial muscle samples to be taken from subjects without impairment of the working capacity. It was then possible to carry out longitudinal training studies and it was shown conclusively that endurance training caused substantial increases in the oxidative potential of both animal and human skeletal muscle (Holloszy, 1973; Saltin, Nazar, Costill, Stein, Jansson, Essén and Gollnick, 1976).

The effects of strength training have not been so widely investigated but Thorstensson (1976) carried out a detailed study and found an increase in muscle mass associated with strength training. The maximal strength of a muscle has been shown to be proportional to the cross-sectional area of the muscle (Ikai and Fukunaga, 1968) and in accordance with this it has been found that athletes trained for extreme strength, such as weight-lifters, displayed larger muscle fibres than untrained

subjects (Edström and Ekblom, 1972; Gollnick, Armstrong, Saubert IV, Piehl and Saltin, 1972; Prince *et al*, 1976; Haggmark, Jansson and Suane, 1978). The fibre type distribution is of importance to isometric strength performance in that those athletes with a higher proportion of Type II fibres have the potential to be isometrically stronger (Tesch and Karlsson, 1978) and this relationship, between the percentage of FT fibres and power performance, becomes increasingly more important as the velocity of movement increases (Coyle, Costill and Lesmes, 1979). The hypertrophy is selective to the Type II fibres (Thorstensson, 1977) with an increase in the fibre area ratio of Type IIA to Type I and Type IIA to Type IIB (Costill, Coyle, Fink, Lesmes and Witzmann, 1979).

The energy pathways of the two types of exercise, strength and endurance, may be altered by both diet and training. Krogh and Lindhard (1920) had established the two main fuels as CHO and lipid, and Hermansen, Hultman and Saltin (1967) found that exhaustion after prolonged heavy exercise occurred simultaneously to the muscles CHO store, glycogen, being depleted. These findings stimulated experiments which looked at the effects of dietary modifications in maintaining the glycogen content of muscle. One method would be to effect a glycogen-sparing metabolism. This has been possible in exercising rats (Rennie, Winder and Holloszy, 1976; Hickson, Rennie, Conlee, Winder and Holloszy, 1977) and in humans (Costill, Coyle, Dalsky, Evans, Fink and Hoopes, 1977) when there are elevated

levels of free fatty acids. A low CHO diet will produce increased FFA by reducing lactate production (Maughan, Williams, Campbell and Hepburn, 1978) and thus reducing the inhibitory effect on lipolysis (Boyd, Giamber, Mager and Lebovitz, 1974). The increased availability of FFA will, in turn, have an inhibitory effect on glycolysis (Newsholme, Randle and Manchester, 1962) and therefore produce a decreased utilisation of glycogen. An alternative method of prolonging the glycogen supplies would be to increase the initial concentration and this can be achieved by giving subjects a high CHO diet after exercise (Hultman, 1967). However, at present there is no evidence available to explain the mechanisms causing this supercompensation of glycogen in skeletal muscle.

It is evident therefore that muscle glycogen levels can be maintained either by a sparing effect of the glycogen or by increasing the amount available for breakdown. Either way, the time taken until exhaustion may be extended.

The utilisation of the substrates in addition to varying with the intensity of exercise varies within the various muscle types. In horses (Lindholm, Bjerneld and Saltin, 1974) it has been demonstrated that maximal speed is required in order to bring most FT fibres into use and that glycogen depletion occurred selectively in the 3 fibre types with FTH and ST fibres being active at exercise intensities up to maximal trotting speeds. Studies in human have shown a similar recruitment pattern

(Gollnick, Armstrong, Saltin, Saubert IV, Sembrowich and Shephard, 1973; Gollnick, Piehl and Saltin, 1974). These results were based on histochemical criteria but have now been confirmed by single fibre analysis (Essén, 1978). From these results it was concluded that the glycogen depletion pattern is mainly dictated by the work intensity but that the mode and duration of the exercise may be important.

Many studies have now been carried out on the effects of training on different parameters of skeletal muscle. These include studies on man (Gollnick *et al*, 1973; Thorstensson, 1976; Henriksson, 1977; Essén, 1978) and horse (Lindholm, 1973; Guy and Snow, 1977a,b; Snow and Guy, 1979, 1980). The effects of detraining (a period of relative inactivity after a period of activity) are less well documented. Faulkner, Maxwell and Lieberman (1972) describe a histochemical study on guinea-pig where detraining after a period of endurance work caused a regression of red fibres compared to white. Further confirmation of a decreased oxidative capacity after endurance training is described in rats (Benzi, Panceri, Bernardi, Villa, Arcelli, D'angelo, Arrigoni and Berté, 1975). An unusual effect, however, has been reported in Thoroughbred horses after 10 weeks of detraining (Guy and Snow, 1977a) where the enzyme levels, after an initial decline in activity, began to increase. No reason could be given for the rebound effect and as yet no further studies have been carried out. Detraining

after strength training results in muscle atrophy (Thorstensson, 1977). Acid hydrolases reflect the degree of skeletal muscle atrophy (Max, 1972) although their activity after acute exercise will also increase if the animals are untrained (Vihko, Salminen and Rantamaki, 1979). The increased activity is concomitant with fibre damage (Vihko *et al*, 1979).

Mitochondrial disruption has already been noted in electron microscopical sections of fatigued muscle (Gollnick and King, 1969) but in this case, the cellular disruption was only noted in the trained animal. The ultrastructural evidence in the latter case, however, still remains controversial (Gale, 1974).

THE EFFECT OF ANABOLIC STEROIDS ON MUSCLE PLASTICITY

It is apparent that males are usually larger and stronger than females. The dependence of these male characteristics on the testes has been known since early times and it was not surprising when Kochakian and Murlin (1935) demonstrated that the testicular hormone testosterone possessed an anabolic action. The natural male hormone's action can be divided into two: a) androgenic, b) anabolic. The androgenic activity promotes the development of the male accessory reproductive glands and the secondary sexual characteristics. The anabolic effect is more general and causes the growth of muscles due to a stimulating effect on the transcription of ribosomal RNA (Rogozkin and Feldkoren, 1979). Much of the early research in this field was carried out on castrate

animals with subsequent testosterone therapy. It was found that not only did species vary in their response to testosterone (Kochakian, 1950) but that individual muscles of the one animal responded differentially (Kochakian, Tillotson, Austin, Dougherty, Haag and Coalson, 1956). The response noted was a growth response in that skeletal muscle atrophy after castration can be reverted by testosterone therapy (Kochakian *et al*, 1956).

Other responses have been evident. Glycogen content has been increased in various muscles of the rat after testosterone treatment. The increase in the female was significant (Leonard, 1952) while smaller increases were seen in the castrate male (Bergamini, 1975). Meyer and Hershberger (1957) found the rise to be pronounced in the 24-72 hour period after which the glycogen content fell off. They suggested that the increase in glycogen was a prerequisite for the increased metabolism necessary for an enhanced protein content. The quantity of protein in any tissue is controlled by the difference between the rate of synthesis and degradation of protein. Rates of protein breakdown have been determined in skeletal muscle under anabolic conditions and it was found that protein breakdown was increased in anabolic as well as catabolic situations (Millward, Bates, Laurent and Lo, 1978). It would appear therefore that muscle growth on administration of steroids would be reflected in an increased protein synthesis. In contrast, the mode of glycogen increase is thought to be due to a glycogen-

sparing effect i.e. a decrease in breakdown (Bergamini, 1975).

These are the main effects of testosterone on skeletal muscle but since 1935 research has been making concerted efforts to find compounds which show a high anabolic effect with a reduced androgenic side effect.

Androgens are 19 carbon steroids with methyl groups at C_{18} and C_{19}. At present changes to produce effective anabolic steroids have fallen into two categories (Figure 1.2):

1. those in which the methyl group at C_{19} is absent,
2. those in which the methyl group is present but which have extra double bonds in their basic structure.

For clinical administration these basic compounds are not used because of their very short duration of activity. To overcome this, esterification of the 17-hydroxyl group yields compounds of longer duration of activity and potentiates their relative activity (Figure 1.3).

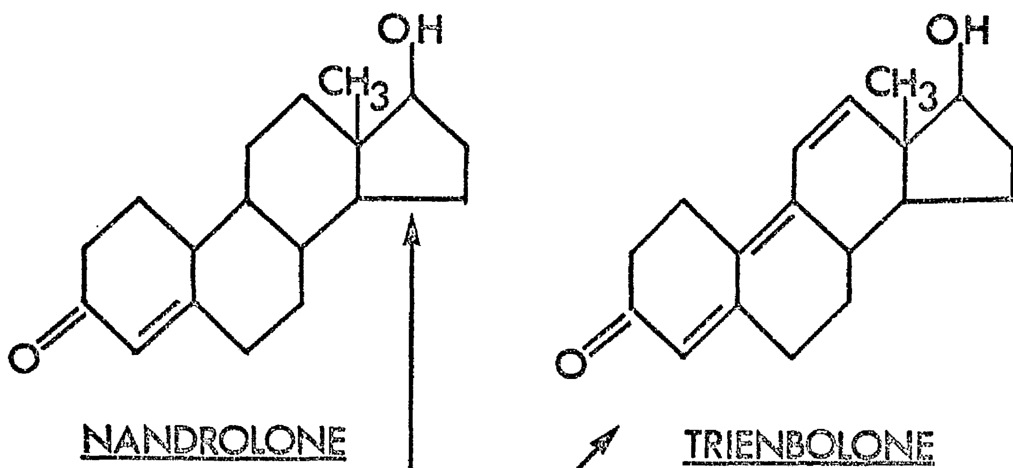
The classical means of determining the ratio of anabolic:androgenic activity is reflected in the myotropic androgenic index (Eisenberg and Gordan, 1950).

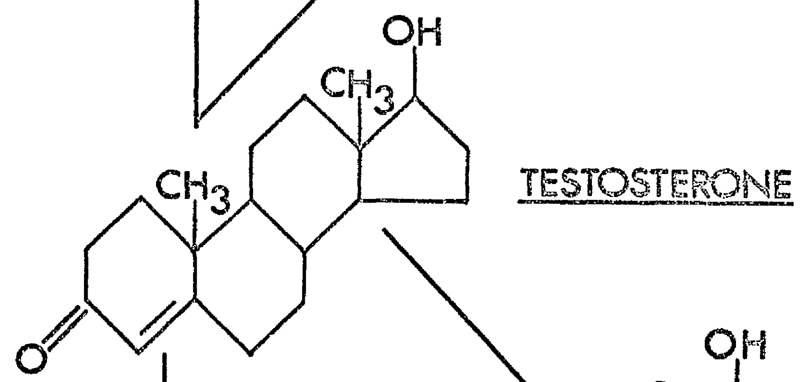
Myotropic-Androgenic index (M/A index)

$$= \frac{\text{wt. of levator ani of treated} - \text{wt. of levator of controls}}{\text{wt. of seminal vesicles of treated} - \text{wt. of seminal vesicles of controls.}}$$

The levator ani was seen as an expression of the anabolic property of the steroid. However extensive

Figure 1.2 Structure of commonly used anabolic steroids in equine practice


NANDROLONE
TRIENBOLONE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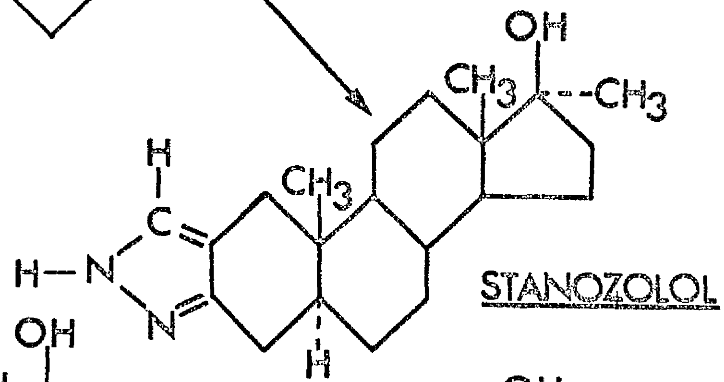
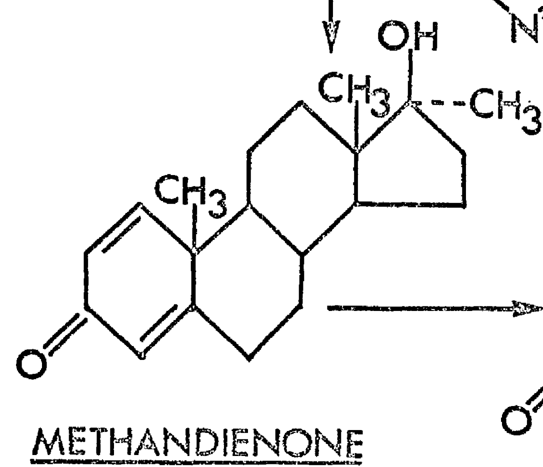

STANOZOLOL


METHANDIENONE

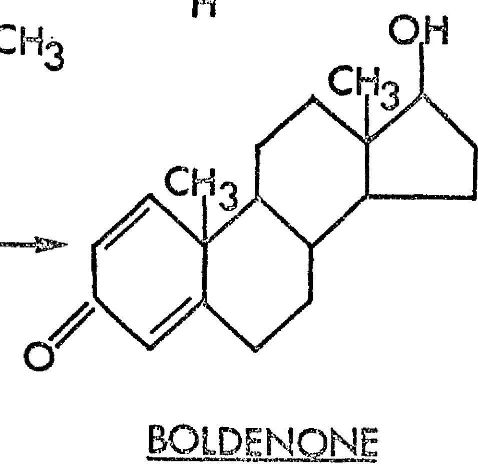
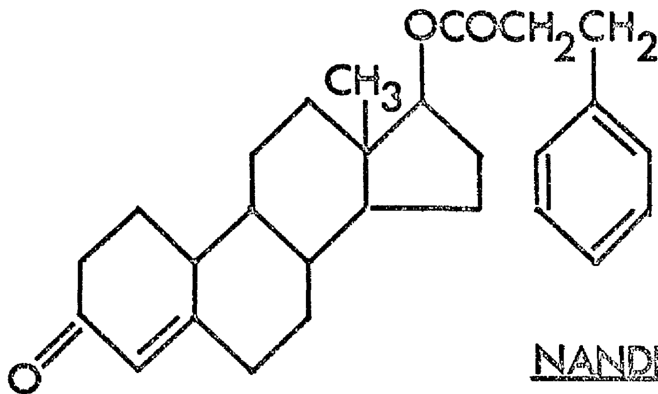
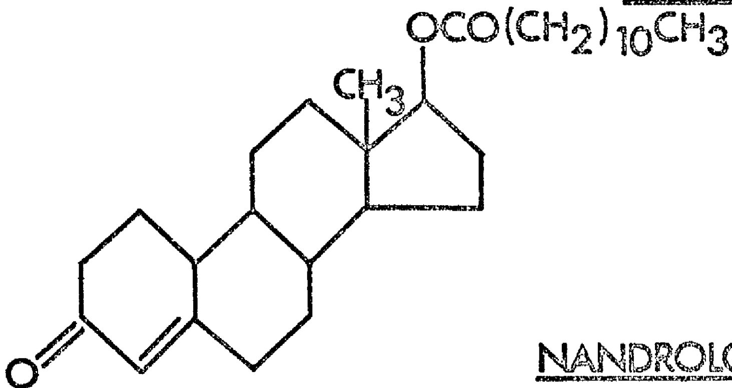

BOLDENONE

Figure 1.3 Effect of different esters on the duration of blood levels of nandrolone. Figures in parenthesis indicate half-life of the compounds in rat plasma.

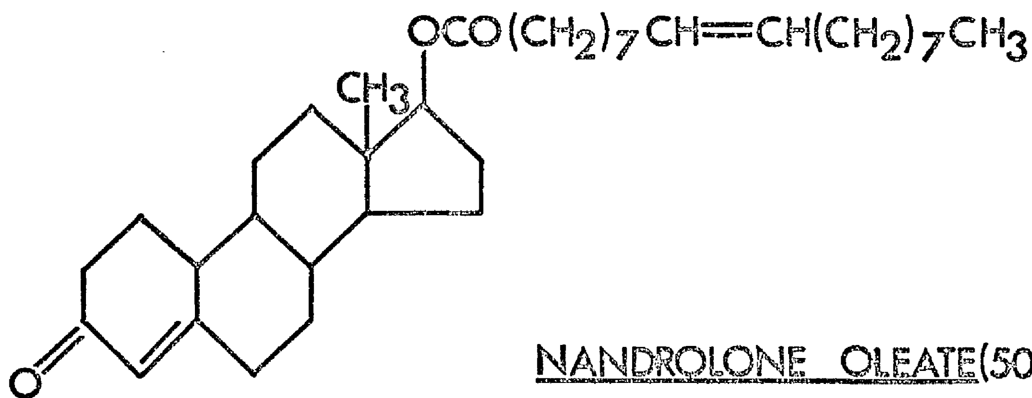
ESTERS OF NANDROLONE



NANDROLONE PHENYL PROPIONATE (25hr)



NANDROLONE LAURATE (130hr)



NANDROLONE OLEATE (504hr)

animal experiments now seem to invalidate the relevance of this muscle as a measure of the myotropic activity (Hervey, Hutchison, Knibbs, Burkinshaw, Jones, Morgan and Levell, 1976). The absence of a standard method for the determination of the M/A index relevant to clinical practices, precludes the quantitative comparison of anabolic steroids being used therapeutically.

Their introduction into sport was based on the experience of an American physician who after several tests on himself was satisfied that an anabolic steroid, taken in conjunction with strength training and a high protein diet, yielded higher gains in muscle strength than in the control situation. Unfortunately he reported his findings in a journal catering for weight-lifters and body-builders. Since that time anabolic steroid usage in sport has become widespread. Further studies on humans have supported the view that steroids combined with a progressive resistance programme results in a greater increase in muscle girth than with exercise alone (Bowers and Reardon, 1972; Ward, 1973; Taylor, Secord and Murray, 1973; O'Shea and Winkler, 1970; Johnson, Fisher, Silvester and Hopkins, 1972; Hervey *et al*, 1976). It has been suggested that the increase in girth may be caused by an increased water retention (Casner, Early and Carlson, 1971) although there are also reports of increased strength and power following administration of the steroids (Ariel, 1972, 1973; Ariel and Saville, 1972a, b, c; Bowers and Reardon, 1972).

Ryan (1976) reviewed many of the publications in this field and it would appear that many of the papers showing an improved performance did not have adequate controls or the experiments were not double-blind. The importance of this latter point is emphasised in one study where a considerable performance improvement was noted when the athletes were given a placebo (Ariel and Saville, 1972).

Two detailed studies have now been carried out on humans (Freed, Banks, Longson and Burley, 1975; Hervey *et al*, 1976). However these results too are contradictory. Both groups were administering methan-dienone (dianabol - CIBA) but Hervey *et al* (1976) found no improvement in performance whereas Freed *et al* (1975) did. Some clue as to the reason for the continuing contradictory evidence may lie in the fact that Freed's control and anabolic-treated group did not undergo similar training programmes and it transpired that the treated group trained more frequently. This alone could result in an improved performance although the reason for the increased ability to train could still be an effect of the anabolic steroids. More consistent results are evident from experiments on laboratory animals. In sprint-exercised and resting male rats treated with dianabol there was no water retention within skeletal muscle, no change in lean body mass and no increase in skeletal muscle glycogen or fibre area (Hickson, Heusner, Van Huss, Jackson, Anderson, Jones and Psaledas, 1976a;

Hickson, Heusner, Van Huss, Taylor and Carrow, 1976b). Treatment again of male rats with this preparation did not show any improvement in strength/power performance (Stone and Lipner, 1978). In accordance with the measurements of glycogen content, glycogenolytic and glycolytic enzymes did not alter in resting or exercising male rats. However the enzymes representing these pathways in resting females increased significantly. This is comparable to the results noted with testosterone on glycogen content (Bergamini, 1975). The increase with the anabolic steroid administration was similar to that seen in a non-treated isometrically exercised group. The increase in enzyme activity in the females was not enhanced further by training (Exner, Staudte and Pette, 1973).

With the purported enhancement of muscle bulk associated with the administration of anabolic steroids it was evident that they might be of advantage in animal production. In addition to using the anabolic action of the androgenic steroids, Heitzman (1976) found that the best growth response in bulls was found by using an oestrogenic steroid. His results on the relationship of anabolic agents with the sex status may be summarized by the following:

The steroid which produces the maximal growth response in different sexes of cattle

Heifers	(female cattle)	androgenic steroid
Bulls	(intact males)	oestrogenic steroid
Steers	(castrate males)	oestrogen ± androgen.

Other reports in beef cattle (Van der Waal, 1975) noted that increasing the dose above therapeutic levels did not enhance the growth but that the response to the steroid reached a plateau.

Anabolic steroids are also being used in horses, this time in connection with their ability to enhance racing performance. Most of the work has been on debilitated animals with reports of increased appetite and improved performance of both Standardbreds and Thoroughbreds (Vigre, 1963; Stihl, 1968).

The improvement noted in racing performance was greatest in geldings, with little or no improvement in stallions (Stihl, 1968) when the anabolic was CIBA 29038-Ba, although improvements have been noted in mares and stallions treated with 17α-methyl-17β-hydroxy-androsta-1,4-dien-3-one (methandienone) (Vigre, 1963). The raised performance was also dependent upon the age of the animal and the distance raced. The best results were in animals aged either 2-3 years old or over 9 years and in those raced over longer distances (2,000 m or more) (Stihl, 1968). The administration of boldenone undecylenate to healthy Thoroughbreds also produced a favourable racing response (Dawson and Gersten, 1978). Although the latter observation was empirically determined increases in muscle mass measurements have been noted in conjunction with an enhanced racing performance in healthy animals treated with 19 nor-testosterone decanoate (Dietz, Mill and Teuscher, 1974).

However no studies have been carried out on muscle composition and it is still unclear whether anabolic steroids do improve racing performance in healthy mature animals.

THE PRESENT STUDY

This study was carried out in order to look at the effects of training and anabolic steroid administration on the skeletal muscle of the horse. The muscle composition of the horse has been well documented in both the Standardbred (Lindholm and Piehl, 1974) and the Thoroughbred (Guy and Snow, 1977; Snow and Guy, 1979; Snow and Guy, 1980). On this basis the following objectives were established for this project.

1) To examine various skeletal muscle parameters relating to administration of an anabolic steroid to healthy resting Thoroughbreds.

2) To examine, in more detail, the effects of training and detraining on the skeletal muscle of the Thoroughbred horse.

3) To study the effect of an anabolic steroid on the skeletal muscle of the Thoroughbred horse throughout a training and detraining programme.

4) To examine some of the effects of acute exercise on skeletal muscle of the Thoroughbred horse.

SECTION 2

GENERAL MATERIALS AND METHODS

ANIMALS USED

All horses used in the anabolic steroid study were geldings.

Number referred to in Text	Breed	Age
1	Thoroughbred	7
2	Thoroughbred	10
3	Thoroughbred	6
4	Heavy Hunter	13
5	Thoroughbred	3
6	Thoroughbred	9
7	Thoroughbred	4

STATISTICS

Conventional statistical methods were used for calculating means ($\bar{x}$), standard deviation (S.D.), standard error of the mean (S.E.M.) and correlation coefficients (r).

Intraindividual differences were tested for significance using the paired t-test and interindividual differences using the Student's 't' test (Snedecor, 1956).

Coefficients of variation for duplicate values were calculated from the formula:

$$C.V. = S.D.\text{ of the difference between double values} \cdot \frac{1}{\sqrt{2}} \cdot \frac{100}{\bar{x}}$$

(Essén, 1978)

When the sample numbers were greater than 2, the coefficient variation was calculated using the formula:

$$C.V. = (S.D./\bar{x}) \times 100$$ (Steel and Torrie, 1976).

Within assay variation was calculated from two samples of the one homogenate, assayed at the start and finish of each batch. Between assay variation was calculated on the first sample of 10 batches.

MUSCLE SAMPLING TECHNIQUES

Muscle samples were obtained from clinically normal geldings using percutaneous needle biopsy (Bergström, 1962). This technique allows serial biopsies to be taken over a period of time without affecting the working capacity of the muscle.

The application of this technique to the horse has been described by Snow and Guy (1976) and the needle is shown in Figure 2.1. After biopsy, the samples were prepared for electron microscopy, histochemistry or biochemistry in the following way:

1) Electron microscopy - A small piece of muscle was immersed in a petri dish containing fixative. The muscle was immediately cut into pieces with a razor blade. The specimens were then transferred to a sample bottle using a pasteur pipette.
2) Histochemistry - The parts of the biopsy sample to be prepared for histochemistry were orientated on filter paper into transverse section under a dissection microscope. The samples were then covered in talcum powder, to prevent ice artifact forming, and immersed in liquid nitrogen. The samples were kept in liquid N_2 until they were removed for cryostat sectioning.
3) Biochemistry - Samples were immediately frozen by immersion in liquid nitrogen using tissue forceps. They were then transferred to plastic vials and stored in liquid nitrogen until analysis.

   Those samples for metabolite analysis were frozen

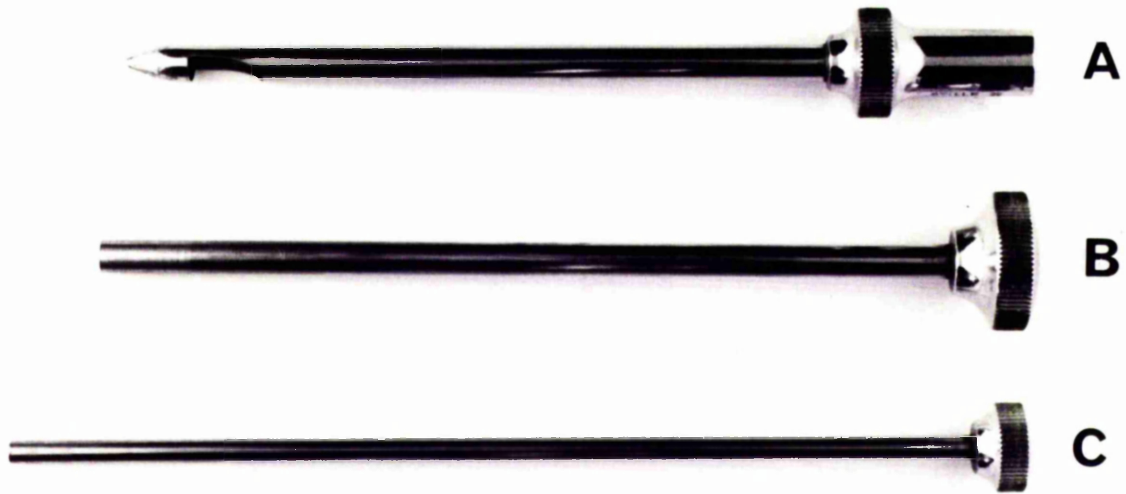


Figure 2.1 Percutaneous muscle biopsy needle

(a) outer needle

(b) cutting cylinder

(c) stylette

directly, while still in the biopsy needle. These samples were also transferred to vials and stored in a liquid N_2 tank.

All samples were taken within a 1 cm square and at a similar depth to minimise possible variation in the muscles.

Control samples taken from horses post-mortem were obtained using the biopsy clamp (Figure 2.2). This instrument holds the muscle at a constant length and was particularly useful in the electron microscopical study.

A large incision was made in the skin, exposing the underlying muscle. Two incisions were then made 2 cm apart and the strip of muscle between was trapped in the jaws of the clamp.

Figure 2.2 Biopsy Clamp

HISTOCHEMISTRY

Samples were removed from liquid N_2 and mounted on a chuck, using O.C.T. (Ames) embedding medium. 10 µm sections were cut on a cryostat (Slee Ltd., London) at -20°C. As it was necessary to obtain good transverse sections for later analysis, the orientation of the initial section was checked by staining with Delafield's Haemotoxylin and Eosin. If the orientation was not satisfactory, then another block was prepared. All subsequent solutions were made up in glass distilled water unless otherwise stated.

SUCCINATE DEHYDROGENASE (SDH)

Paul and Sperling (1952) demonstrated a direct relationship between estimates of mitochondrial density and the oxidative capacity of a variety of muscles from different species. The enzyme succinate dehydrogenase (SDH) is believed to be entirely intramitochondrial (Roodyn, 1967) and could therefore be used as an indication of the mitochondrial density. The histochemical visualisation of SDH is based on the reduction of a diformazan, nitro blue tetrazolium (NBT) by the hydrogen ions released when succinate is converted to fumarate. It is possible that deposition of diformazan may occur outwith the sites of SDH activity, such as lipid droplets (Hitzeman, 1963). However this should have little effect on the comparison between individual fibres. It is also possible that the SDH activity of mitochondria from different fibres may vary (Blancher, 1964) but at incubation times of more than 20 minutes the stain should

depend on mitochondrial density, rather than on actual level of SDH activity (Davies and Gunn, 1972).

The activity of SDH declines significantly within hours at room temperature (Essén, Jansson, Henriksson, Taylor and Saltin, 1975). Sections to be stained for SDH were therefore kept in the cryostat at $-20^{o}C$ and were always stained the same day as sectioning.

Method

The method was based on that of Nachlas, Walker and Seligman (1957)

Reagents:

1) Potassium phosphate buffer 100 mM pH 7.6
2) Sodium succinate 100 mM
3) Nitro-blue tetrazolium 1 mg/ml dist H_2O
4) 4% buffered formaldehyde pH 7.0
5) Incubation medium - Equal volumes of phosphate buffer and sodium succinate, with an equal volume of nitro-blue tetrazolium solution (1:1:2).

Staining protocol:

1) Incubate $37^{o}C$	25-30 min
2) Wash in saline (1M)	
3) Leave to dry at $25^{o}C$	1 hour
4) Fix in formaldehyde	10 min
5) Drying in 15% ethanol	5 min
6) Mount in glycerine jelly	

Diformazan granules occurred as blue dots indicating mitochondrial density. From this it was possible

to classify high and low mitochondrial fibres as high oxidative (HO) and low oxidative (LO).

The slide was viewed on a projection screen and the relative proportions of HO and LO fibres were determined from not less than 100 fibres.

Variation of the percentage of HO types throughout the length of the muscle was determined for the middle gluteal and biceps femoris in a horse after post-mortem. Four well-distributed sites gave coefficients of variation of 4.5% and 4.2% respectively for each muscle.

Biopsy samples, taken from 4 resting horses on two different dates gave a variation of 2.2% in the middle gluteal and 2.3% in the biceps femoris.

The variation due to sampling a different piece of muscle, from the same incision, on a given date was only slightly less, 2.0% (n = 8) in the middle gluteal and also 2.0% (n = 8) in the biceps femoris.

Personal counting error had a coefficient of variation of 0.7% (n = 10).

MYOSIN ADENOSINE TRIPHOSPHATASE (MYOSIN ATP-ASE)

Bárány (1967) has reported that the specific activity of myosin ATP-ase is correlated with the contraction time of muscle and Guth and Samaha (1969) demonstrated that actomyosin ATP-ase measured biochemically is correlated with the histochemical myofibrillar ATP-ase. Histochemical measurement was used in this study to distinguish between the slow-contracting (ST) or Type I and fast-contracting (FT) or Type II fibres.

The method used was essentially that of Padykula and Herman (1955).

A modification to the above method was that preincubation was at pH 10.2 for 15 min at 37°C (Essén *et al*, 1975). This reduced the contraction of fibres often seen with pH 9.4 preincubation. The sections prepared in this way were still clearly distinguishable into two types.

Method

Reagents:

1) Glycine buffer pH 10.2

   per 100 ml solution : 375 mg glycine

   441 mg calcium chloride ($2H_2O$)

   292 mg NaCl

2) 0.05M Tris buffer pH 9.4

3) Incubation medium - 0.2M calcium chloride in 0.05M Tris adenosine-5-triphosphate, disodium salt. Adjust to pH 9.4 with 0.1M NaOH. Freshly prepared and filtered before use.

4) 1% calcium chloride

5) 2% cobaltous chloride

6) 1% ammonium sulphide

Staining protocol:

1) Preincubation glycine buffer 15 min at 37°C

2) Wash in Tris buffer

3) Incubation medium 30 min at 37°C

4) Wash 3 x in calcium chloride 2 min each wash

5) Cobaltous chloride 3 min

6) Wash 3 x in dist. H_2O

7) Develop in fresh ammonium sulphide 1 min

8) Wash in tap water, dehydrate, clear and mount

Variation of the ATP-ase stain throughout the muscle was calculated in a similar fashion to the SDH stains. The values were:

	Middle gluteal	Biceps femoris
Throughout the length of the muscle	4.3%	4.6%
Different biopsy dates	3.5%	2.8%
Different muscle specimen, same incision	1.9%	2.1%
Personal counting error	0	0

FIBRE CLASSIFICATION

The histochemical profile of equine skeletal muscle has been well studied (Lindholm, 1974; Guy and Snow, 1977a; Snow and Guy, 1976; Snow and Guy, 1980). In these studies the fibres were classified as fast twitch low oxidative (FT), fast twitch high oxidative (FTH) and slow twitch high oxidative (ST).

The percentage of ST fibres can be calculated from the myosin ATP-ase stain. The SDH stain further divides the fibres into high and low oxidative. The low oxidative fibres are all FT fibres whereas the high oxidative includes both the FTH plus the ST.

The % FTH = 100 - (% FT + % ST).

Subdivision of the high myosin ATP-ase pH 9.4 fibres can also be carried out by preincubating at various

acid pHs (Brooke and Kaiser, 1970). Two subtypes IIA and IIB have been described in the horse (Snow and Guy, 1980). These workers found that the subtypes using acid preincubation were not completely identical to the subtypes based on metabolic properties. This has also been found in man (Saltin, Henriksson, Nygaard, Andersen and Jansson, 1977).

In the present study, continuing from the earlier equine work, the nomenclature is based on the metabolic properties of the muscle fibres and are termed FT, FTH and ST (Figure 2.3).

AMYLASE - P.A.S.

Capillary density has been calculated in the skeletal muscle of man using a Periodic Acid-Schiff reaction (Hecht, 1958). Transverse frozen sections are emptied of glycogen by incubating with α-amylase. Subsequent oxidation is carried out in periodic acid and the section stained with Schiff's reagent. This method most likely stains a mucopolysaccharide in the muscle and the capillary basement membrane. Capillary densities calculated with this method are in good agreement with electron-microscopical data (Andersen, 1975). Furthermore it is indicated that by using this method, the counting of capillaries is not affected by the number of open or closed capillaries i.e. the blood flow through the muscle (Hermansen and Wachtlova, 1971).

Figure 2.3 Serial section of horse muscle stained

for (a) myosin ATP-ase (pre-incubation 10.2)

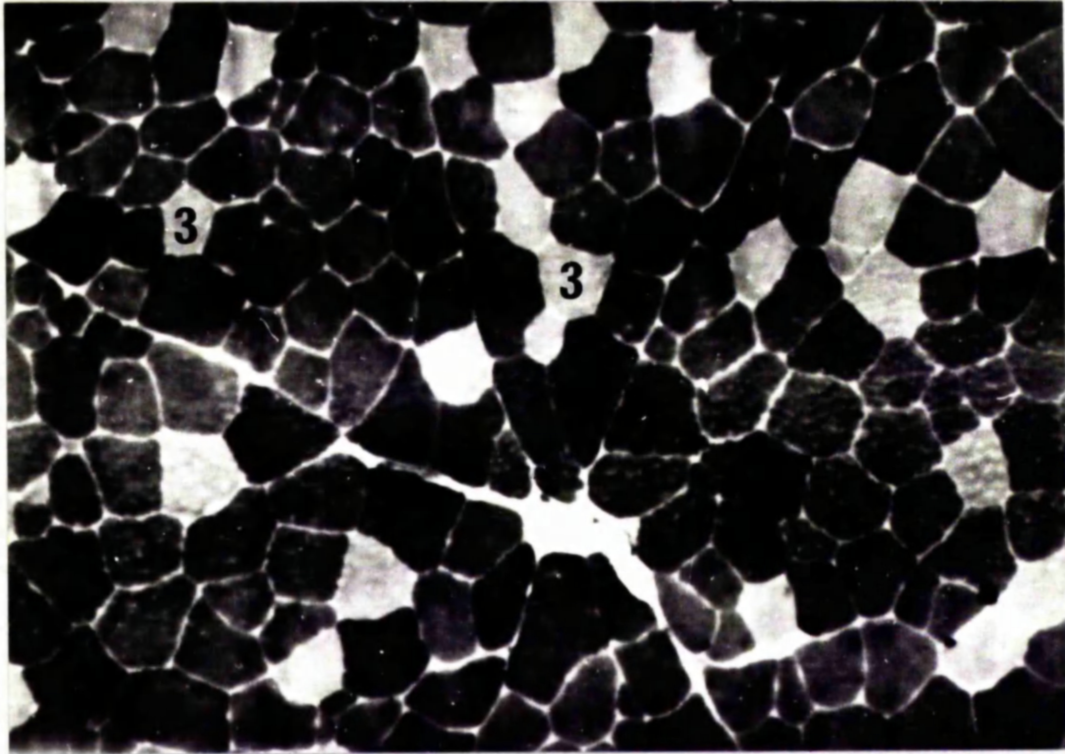
(b) SDH

Mag: x 160

1 = FT

2 = FTH

3 = ST


3
3

2
3 2
1
3 2
1
1

Method

Reagents:

1) Carnoy's fixative - 60 ml ethanol
30 ml chloroform
10 ml M acetic acid

2) 50% α-amylase from solution

3) 1% periodic acid

4) Schiff's reagent

Staining protocol:

1) Fix in Carnoy's 21°C 10 min

2) Rinse in redistilled H_2O

3) Incubate in α -amylase 37°C 30 min

4) Rinse in redistilled H_2O

5) Incubate periodic acid 21°C 10 min

6) Stain with Schiff's reagent 21°C 30 min

7) Dehydrate, clear and mount.

A complete description of capillary supply to a tissue can only be expressed in a three-dimensional system. However since the capillary arrangement in mammalian muscle is, in general, quite regular with capillaries running parallel to the muscle fibre (Krogh, 1918-1919) it is possible to quantify the capillary supply on counts made from transverse sections (Spalteholtz, 1888; Krogh, 1918-1919; Hermansen and Wachtlova, 1971; Andersen, 1975). The counts have been expressed in several ways: cap. mm^{-2} (Krogh, 1918-1919) cap. $fibre^{-1}$ (Smith and Giovacchini, 1956), number of capillaries around fibres (Romanul, 1965) and capillaries around each

fibre relative to fibre area (Ingjer, 1979a, b).

In this study the amylase-PAS sections were serially matched to SDH-stained sections and the number of capillaries around each fibre type, HO or LO, were counted from photomicrographs magnified 400x.

Capillaries were then expressed relative to the fibre area of the HO and LO fibres (see section on fibre areas) (Figure 2.4).

FIBRE AREAS

Published data on fibre areas has, in general, been carried out on sections stained for myosin ATP-ase activity (Tesch and Karlsson, 1978; Ingjer, 1979a, b). Inconsistent contraction of fibres stained with myosin ATP-ase is common even if precautionary measures are taken (vide supra). Area measurements on sections stained with amylase-PAS give an approximately 5% larger area than measurements on sections stained for ATP-ase (Andersen and Henriksson, 1977). For this reason myosin ATP-ase sections were not used for fibre area analysis in this study.

On comparison of serial sections, there was no significant difference between areas calculated on the SDH sections compared to the amylase-PAS. Furthermore the variation produced by each stain (mean value for 10 duplicate biopsies taken from the same incision, were compared) showed that the SDH sections gave a value of 5.9% for HO fibres and 9.8% for LO fibres. Similar comparison of the amylase-PAS sections showed a variation of 8.6% and 10.3% respectively.

Figure 2.4 Serial sections of horse muscle stained for

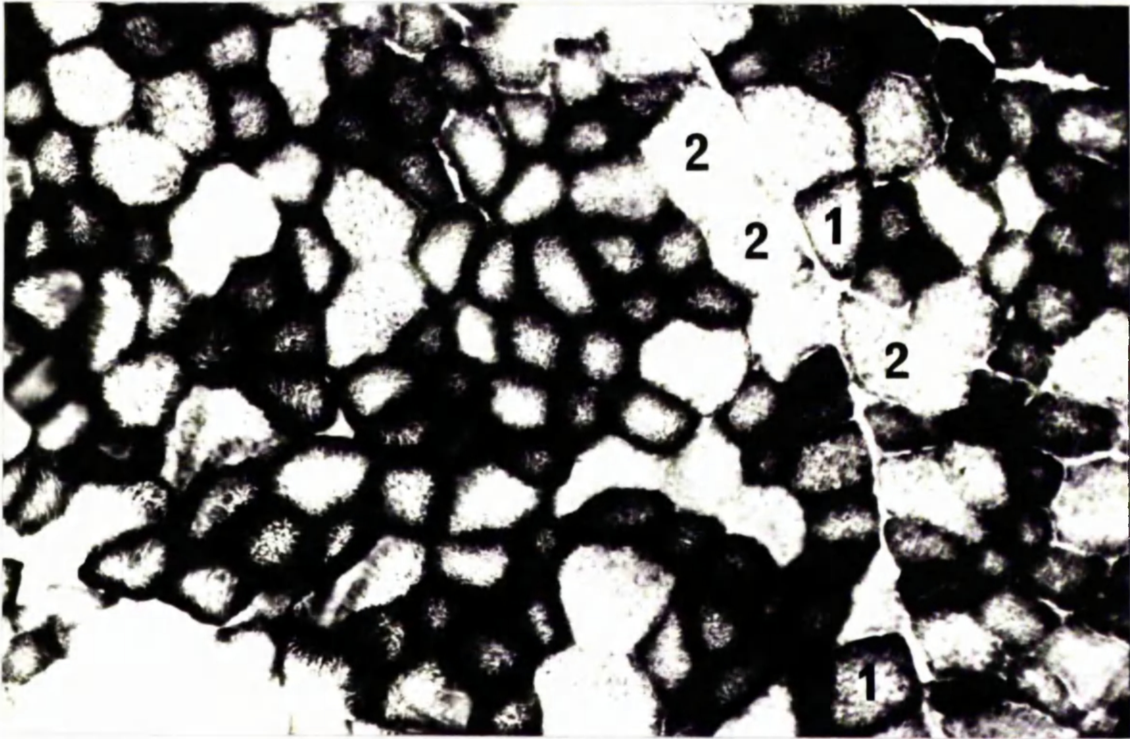
a) SDH

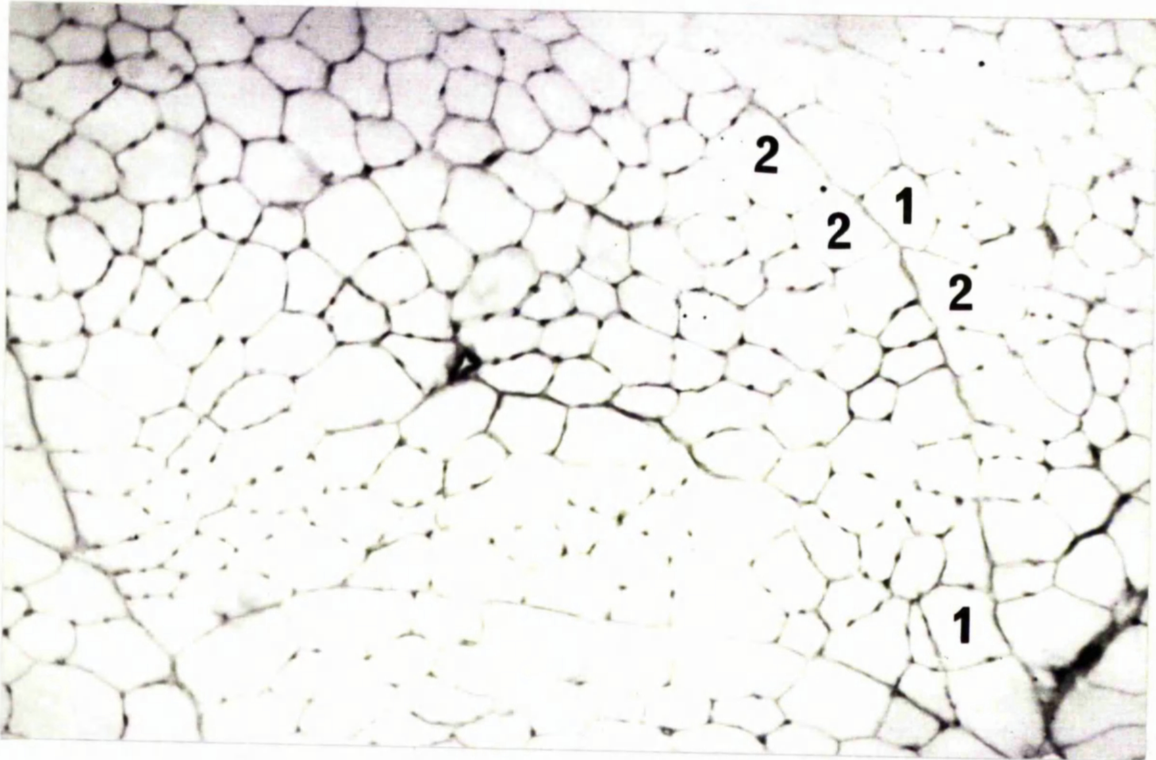
b) Amylase P.A.S.

x 160

1 = HO fibre

2 = LO fibre


2
2
1
2
1


2
2
1
2
1

As there was little preference as to which stain should be selected for the measurement of fibre areas, on the basis of the variation, the SDH sections were chosen for fibre area analysis.

Photomicrographs of the sections were enlarged 400x and the areas of the HO and LO fibres calculated using a M.O.P./AM02 modular image analysing system (Kontron).

BIOCHEMISTRY

ENZYMES

The majority of measurements on enzyme activity in this study utilize a nicotinamide coenzyme in an oxidation/reduction reaction directly or the reaction can be linked to a dehydrogenase system which does. Since the reduced forms of the nicotinamide coenzymes (NADH/NADPH) have an absorption peak at 340 nm, while the oxidised forms (NAD^+/$NADP^+$) do not, these enzymes can all be measured by the rate of change of extinction at 340 nm. The activity of the enzyme is proportional to the rate of change of the optical density

i.e. $E.A = \Delta Emin^{-1}$ 1000 $(M.E.C.)^{-1}$ total tissue dilution

E.A. = enzyme activity

ΔE = change in optical density

M.E.C. = the molar extinction coefficient of the compound being measured.

At 340 nm the M.E.C. of NAD is 6.22 cm^2 $mole^{-1}$.

The enzyme assays were all carried out at $37^{o}C$ as recommended by the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974).

The following enzymes were measured on an LKB 8,600 reaction rate analyser:

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), 3 hydroxyacyl-Coa-dehydrogenase (HAD), lactate dehydrogenase (LDH) and phosphofructokinase (PFK).

A further 3 enzymes, aldolase (ALD), citrate

synthase (CS) and cytochrome oxidase (cyt-ox) were measured on a Pye Unicam SP 8000 U.V. spectrophotometer.

β-Glucuronidase (β-Gluc) was measured on a Perkin-Elmer 1,000M fluorimeter.

All assays were carried out in cuvettes of pathlength 1 cm.

Chemicals were obtained from 2 main sources, Boehringer Mannheim (London) and Sigma Company (London). Where possible commercial kits were used to standardise reaction conditions and improve the precision and reproducibility of the assay. To check the stability of the reagents between assays a precipath (Boehringer) sample was assayed where possible. In addition a control muscle sample was used throughout the total analyses.

The between assay and within assay variations are given in Table 2.1.

The admixture of blood, adipose and connective tissue which is sometimes found in a muscle sample can produce large variations in enzyme determinations. This can be reduced by freeze-drying the muscle (Speedivac Pearse, Tissue Dryer Mk. I, Edwards High Vacuum) at -40°C and 0.05 torr and powdering it with an agate pestle and mortar thus removing any of the extra myofibrillar components.

All muscle samples for enzyme analysis were prepared in this way and were not significantly different from comparable wet weight samples.

TABLE 2.1 Concentration of substances used in the various enzyme assays with their between assay and within assay variation (coefficient of variation)

Enzyme	Buffer	pH	Reagents	Amount of dry tissue (mg)	Between assay variation	Within assay variation
AST	80mM Phosphate	7.4	200 mM L-aspartate 0.18mM NADH MDH >0.6 U ml^{-1}, LDH >1.2 U ml^{-1} and 12 mM α-oxoglutarate	0.02	5.6	1.6
ALT	80mM Phosphate	7.4	800mM L(+)-alanine 0.18mM NADH 18mM α-oxoglutarate and LDH >1.2 U ml^{-1}	0.10	3.7	2.4
CS	100mM Tris-HCl	8.1	0.1mM DTNB, 1.0mM acetyl CoA and 0.5mM oxaloacetate	0.05	0.9	0.7
LDH	50mM Phosphate	7.5	0.18mM NADH, 0.6mM pyruvate	0.01	2.5	1.2
ALD	100mM Phosphate	7.0	5mM fructose-1,6-diphosphate, 0.3mM iodoacetate, 0.12mM NADH, GDH >0.13 U ml^{-1} and TIM >4 U ml^{-1}	0.05	2.5	1.6
HAD	50mM Imidazole	7.0	0.5mM EDTA 0.124mM NADH and 0.0275mM acetoacetyl CoA	0.01	2.7	1.7
PFK	0.2M Tris-HCl	8.25	0.02M ATP pH 7.0, 0.02M $MgSO_4$ 0.76mM NADH, 0.025M F-6-P, 11mM DTT, 0.5M KCl 10 mg/ml Aldolase, 10 mg/ml TIM/Glycerol PO_4	0.01	3.1	1.4
β-Gluc	0.1M Acetate	3.5	1.5mM 4-methylumbelliferone, 1M Na_2CO_3	0.1	1.9	1.5
Cyt.Ox.	0.03M Phosphate	7.4	1.2M Na Dithionite Few grains of Potassium ferricyanide	0.1	1.3	0.6

HOMOGENATION SOLUTION

In all cases the muscle samples for enzyme analysis were hand homogenised in a solution of 150 mM KCl, 50 mM $KHCO_3$ and 6 mM ethylene diamino tetra acetic acid (EDTA) in water, pH 7.4. Protein concentration was enhanced with 1% horse serum albumin. Solutions were made up to give 1 mg/1 ml solution. Suitable dilutions of this homogenate were used to measure the enzyme activities.

Homogenation may not be sufficient for measuring the activity of those enzymes present within the mitochondrial matrix. Triton X-100 was therefore added to the homogenising medium of some samples to make sure that all the enzymic activity had been completely released. Two concentrations of Triton X-100 were used: 0.1% and 1%. There was no effect noted in any of the enzymes studied (Table 2.2).

The stability of the enzymes, in the homogenising medium when kept on ice, is given in Table 2.3. The enzymes which were not stable for 3 hours are referred to individually in the text. All solutions were made up in glass distilled water unless otherwise stated.

CITRATE SYNTHASE (C.S.:E.C.4.1.3.7)
(Citrate oxaloacetate-lyase-CoA-acetylating)

The citrate synthase reaction is the primary pacemaker step of the tricarboxylic acid cycle (Lehninger, 1975), and its reaction proceeds far in the direction of citrate formation.

TABLE 2.2 The effect of Triton X-100 on several muscle enzymes of the horse. Each value is the mean of four samples

Enzyme	Homogenate	Homogenate + 0.1% Triton X-100	Homogenate + 1% Triton X-100
CS μmole/min. g dry wt. tissue	10.7	10.6	10.6
AST μmole/min. g dry wt. tissue	496	487	494
ALT μmole/min. g dry wt. tissue	82	82	82
LDH μmole/min. g dry wt. tissue	1830	1850	1863
HAD μmole/min. g dry wt. tissue	163	164	160
β-Gluc mmole substrate hydrolysed/ 60 min. g dry wt. tissue	0.8	0.8	0.8
Cyt. Ox. Δ log (ferrocytochromec) min. for a 1:10,000 dry wt. tissue dilution	13.6	13.7	13.6

TABLE 2.3 The effect of 3 hours in homogenising medium on several enzymes in horse skeletal muscle.
Each value is the mean (μmoles/min. g dry weight tissue) of 3 homogenates

Minutes in homogenate	CS	AST	ALT	LDH	ALD	HAD
0	10.5	860	791	2230	420	274
30	10.5	790	786	2146	410	270
60	10.0	840	780	2240	450	270
90	10.5	840	780	2180	420	274
120	10.5	850	780	2220	430	270
180	10.5	840	780	2260	430	274

The enzyme catalyses the reaction:

$$\begin{array}{c} CH_3 \\ | \\ C = O \\ | \\ SCoA \end{array} + \begin{array}{c} C = O \\ | \\ C\text{-}COOH \\ | \\ CH_2\text{-}COOH \end{array} + H_2O \xrightarrow{C.S.} HO\text{-}\begin{array}{c} CH_2COOH \\ | \\ C\text{-}COOH \\ | \\ CH_2COOH \end{array} + HSCoA$$

Acetyl-CoA + Oxaloacetate Citrate

Method

The activity of citrate synthase was followed by measuring the appearance of the free SH group of the released CoASH.

The free SH can be reacted with 5,5'-dithiobis-(2-nitro-benzoate) (DTNB) (Ellman's Reagent - Srère, Brazil and Gonen, 1963).

$$O_2N\text{-}C_6H_3(COO^-)\text{-}S\text{-}S\text{-}C_6H_3(COO^-)\text{-}NO_2 + RSH$$

$$\downarrow$$

$$O_2N\text{-}C_6H_3(COO^-)\text{-}S\text{-}S\text{-}R + S^-\text{-}C_6H_3(COO^-)\text{-}NO_2 + H^+$$

This reaction is easily followed on a spectrophotometer at 412 nm where the mercaptide ion has a strong absorption. The method used was based on that of Srère (1969).

Reagents:

1) Coenzyme A - 5.2 mg + 2 ml H_2O + 0.006 ml acetic anhydride + 0.03 ml 3M $KHCO_3$
2) DTNB - 2 mg in 5 ml Tris HCl pH 8.1
3) Oxaloacetic acid - 2.46 mg in 0.2 ml Tris HCl pH 8.1 + 1.8 ml dist. H_2O.

Assay: 100 µl DTNB
100 µl AcCoA
0.7 ml dist. H_2O
50 µl sample.
Start reaction with 50 µl oxaloacetate.
Read at 412 nm on spectrophotometer.

The standard curve was established using cysteine as the SH donor. The regression line of the sulphydryl groups (cysteine) against extinction was $y = 0.0061\,x - 0.002$ and was linear over the range 0-100 µmoles of -SH group ($r = 0.998$).

ASPARTATE AMINO TRANSFERASE (AST; E.C. 2.6.1.1)
(L-aspartate:2 oxoglutarate aminotransferase)

Aminotransferases catalyse transamination reactions. These reactions involve the interconversion of a pair of amino acids and a pair of keto acids. As the reaction is a freely reversible process transaminases function both in amino acid catabolism and biosynthesis. AST, an aminotransferase, present in the cytosol and mitochondrial matrix, catalyses the following reaction (Lehninger, 1975):

$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ CHNH_3^+ \\ | \\ COO^- \end{array} + \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ C=O \\ | \\ COOH \end{array} \underset{}{\overset{AST}{\rightleftharpoons}} \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ C=O \\ | \\ COOH \end{array} + \begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_3^+ \\ | \\ COO^- \end{array}$$

Aspartate + 2-oxoglutarate Oxaloacetate Glutamate

Method

The most widely used method of determining AST activity involves the measurement of the oxaloacetate formed from aspartate and 2-oxoglutarate by an enzymic indicator reaction with malate dehydrogenase and a reduced nucleotide (NADH). The indicator reaction allows the conversion of oxaloacetate to malate.

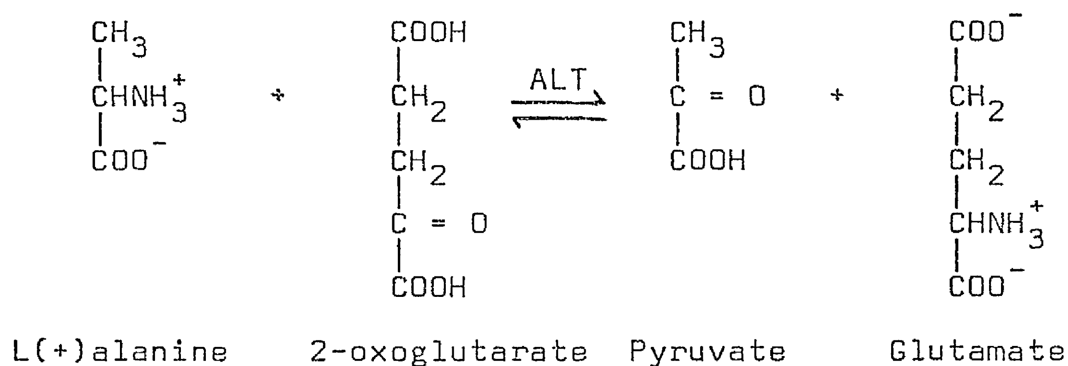
$$\begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ C=O \\ | \\ COOH \end{array} + NADH \overset{MDH}{\rightleftharpoons} \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CHOH \\ | \\ COOH \end{array} + NAD^+$$

Oxaloacetate Malate

The oxidation of NADH, which is proportional to the amount of oxaloacetate formed was measured by the decrease in the optical density at 340 nm using a Boehringer kit No. 124427. The U.V. method used was based on that of Bergmeyer (1974).

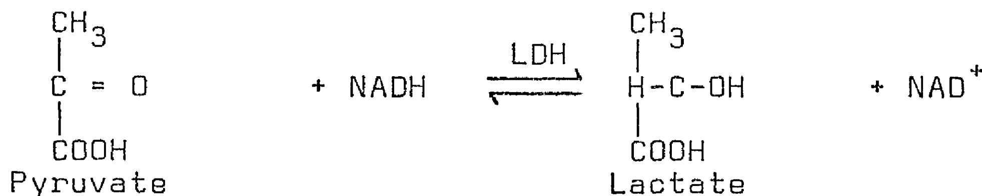
ALANINE AMINOTRANSFERASE (ALT; E.C. 2.6.1.3)
(L-alanine:2-oxoglutarate aminotransferase

This is also a transamination reaction and is present again in the cytosol and mitochondria. The reaction catalyses:



Method

The most commonly used method of measuring this enzyme's activity is based on the pyruvate formed from the alanine and 2-oxoglutarate. The indicator enzyme in this case is lactate dehydrogenase and the method is based on Bergmeyer (1974).

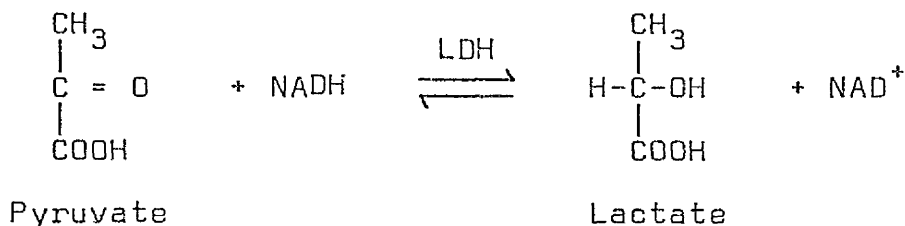


A Boehringer commercial kit No. 124575 was used.

LACTATE DEHYDROGENASE (LDH:E.C.1.1.2.7)
(L-lactate:NAD oxidoreductase)

Lactate dehydrogenase catalyses the last step in glycolysis, the reduction of pyruvate to lactate. The isoenzyme M_4 which is predominant in LDH of skeletal muscle has a relatively high rate at which it reduces pyruvate to lactate. As the equilibrium favours the production of lactate, pyruvate was used as the substrate.

LDH catalyses the reaction:



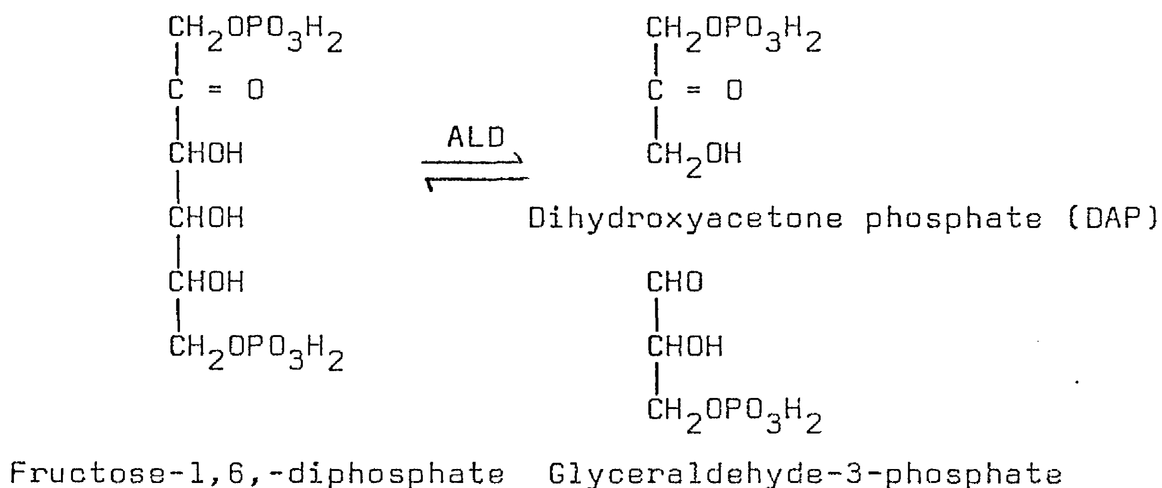
Method

The method used to measure LDH activity was based on that of Wroblewski and La Due (1955).

The homogenate was diluted 1:10 and LDH activity measured using a Boehringer commercial test kit No. 124915.

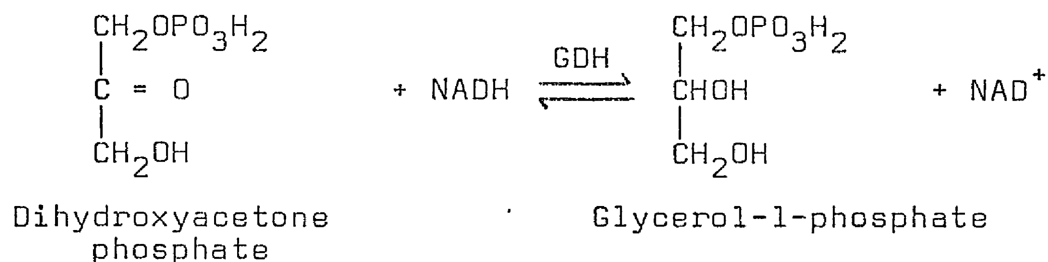
ALDOLASE (ALD: E.C.4.1.2.7)
(Fructose - 1,6-diphosphate D-glyceraldehyde-3-phosphate lyase)

Aldolase catalyses the cleavage of a number of ketose di- and mono-phosphates. In each case, however, dihydroxyacetone phosphate is one of the products. The mechanism of action of aldolase has been extensively studied and from the aspects of comparative biochemistry aldolase is one of the best characterised enzymes. The substrate used in this case was fructose - 1,6-diphosphate and the reaction was



Method

Only one of the two triose phosphates, namely glyceraldehyde-3-phosphate, can be directly degraded in the further reactions of glycolysis. However, the two triose phosphates are interconverted by triosephosphate isomerase (TIM). The equilibrium lies towards the DAP and this is reduced with glycerol-1-phosphate dehydrogenase (GDH) and NADH in the following indicator reaction which is quantitative:



Dihydroxyacetone phosphate — Glycerol-1-phosphate

The method was based on that of Anderson (1975).

Reagents:

1) Phosphate buffer 0.1M
2) NADH 2.5 mg/ml
3) GCH 85 U/ml
4) TIM 2,500 U/ml
5) Iodoacetate 0.01M
6) Fructose-1,diphosphate 0.15M

Reaction Mixture: per 10 assays:

1) Buffer 26.5 ml
2) NADH 1.0 ml
3) GDH 0.01 ml
4) TIM 0.012 ml
5) Iodoacetate 1 ml

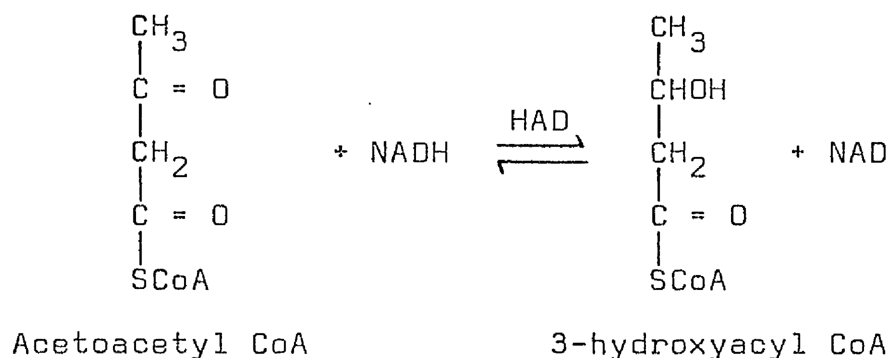
Assay:

Reaction mixture	2.85 ml
Sample	50 µl

Start reaction with 100 µl fructose-1,6-diphosphate.

3-HYDROXYACYL COENZYME A DEHYDROGENASE (HAD:E.C.1.1.1.35)
(L-3-hydroxyacyl-CoA:NAD oxidoreductase)

HAD catalyses the second dehydrogenation step in fatty acid oxidation. It is present in the mitochondrial matrix (Lehninger, 1975) and catalyses the reversible reaction:



Acetoacetyl CoA 3-hydroxyacyl CoA

<u>Method</u>

At pH 7.0 the reaction favours the formation of 3-hydroxyacyl CoA while the reaction is reversed at a pH greater than 9.0. In this study the reaction was assayed in the direction of acetoacyl CoA → 3 hydroxyacyl CoA.

The method used was that of Essén *et al* (1975) with reduced reaction volumes to allow the method to be carried out on an LKB reaction rate analyser.

Reagents:

1) Imidazole buffer	83 mM pH 7.0
2) NADH	11.3 mg/10 ml
3) EDTA	6 mM
4) Acetoacetyl CoA	Stock of 1 mg/ml (kept at

-90°C). Dilute 0.6 ml stock
to 2 ml dist. H_2O.

Reaction mixture: Per 10 assays

1) Imidazole buffer	0.6 ml
2) NADH	0.1 ml
3) EDTA	0.1 ml
4) H_2O	0.1 ml

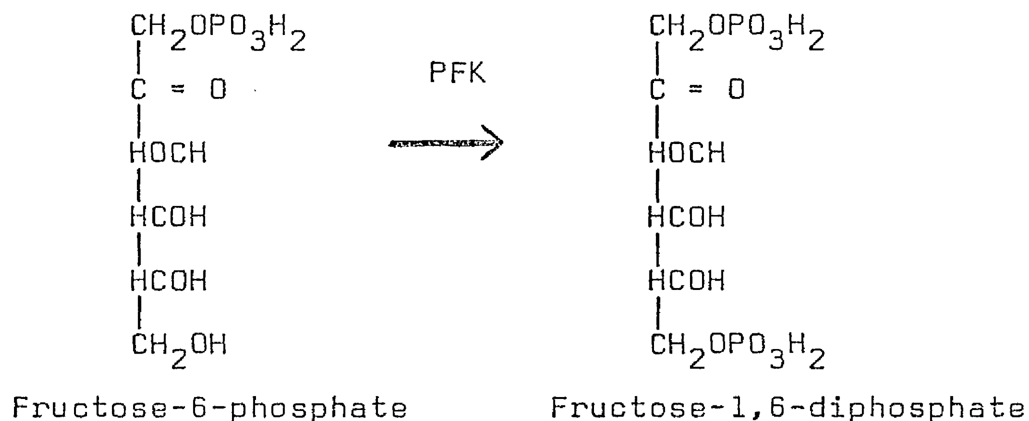
Assay:

Reaction mixture	0.9 ml
Sample	0.1 ml (1:10 homogenate dilution)

Start reaction with 0.1 ml acetoacetyl CoA.

PHOSPHOFRUCTOKINASE (E.C.2.7.1.11)
(ATP d-fructose-6-phosphate-1-phosphotransferase)

Phosphofructokinase is the most important control point in the glycolytic sequence (Lehninger, 1975). Its kinetic behaviour, though very complex, is extraordinarily well adapted for the regulation of glycolysis. The reaction is irreversible. The enzyme catalyses the reaction:



Method

The method of measuring the activity of the enzyme in this study is essentially the method described by

Ling, Paetkau, Marcus and Lardy (1966). The authors used the method for assays of PFK in partially purified extracts of rabbit skeletal muscle but it has been found to be satisfactory with crude tissue extracts. The Mg^{2+} content has been reduced to an ATP/Mg^{2+} ratio of 1:1 in this study as large excesses of magnesium may be inhibitory (Mansour, 1972). The reaction volumes have been reduced to enable measurement on the LKB reaction rate analyser.

Reagents:

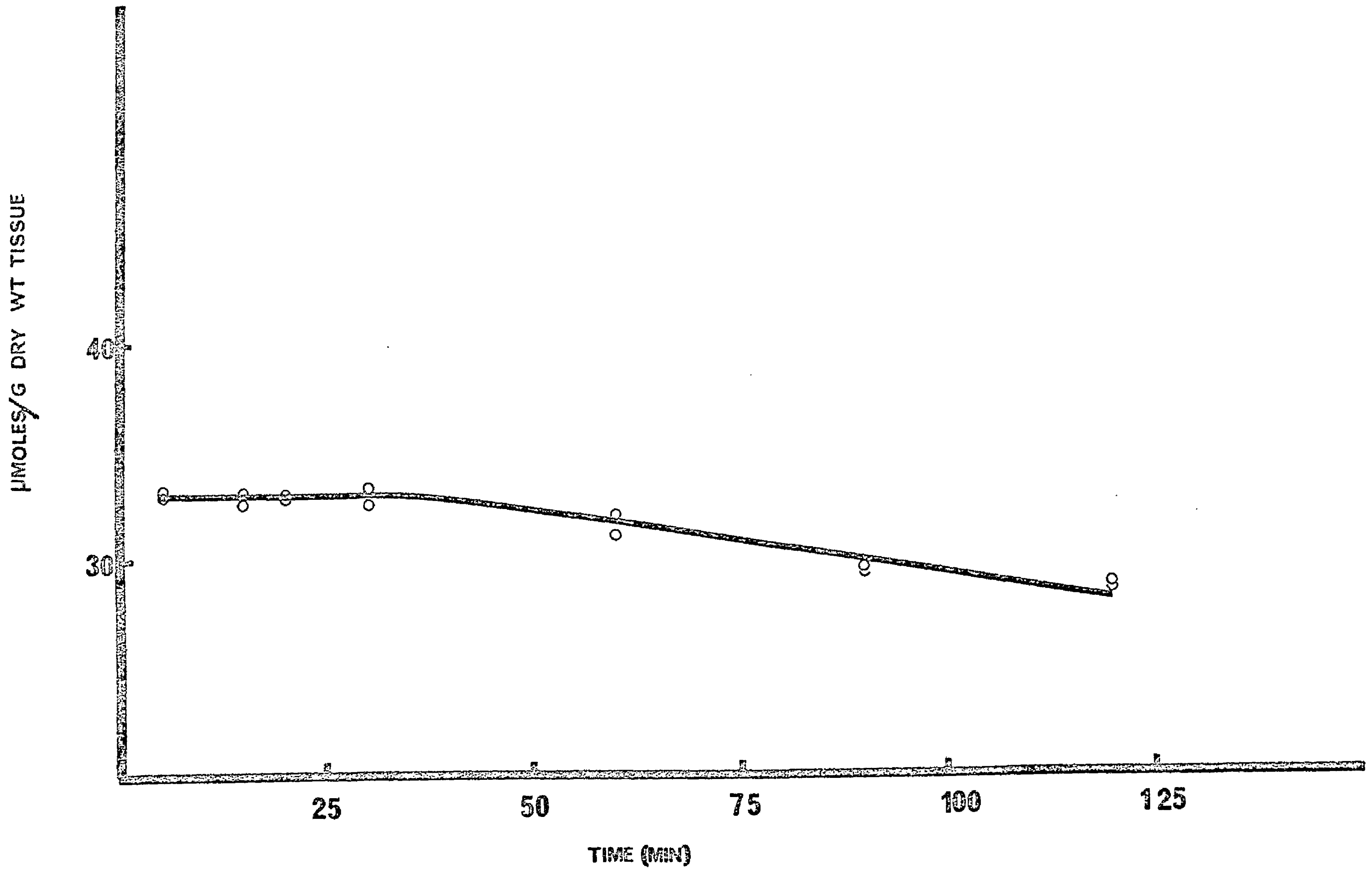
1)	Tris HCl	0.2M pH 8.25
2)	ATP	0.02M pH 7.0
3)	$MgSO_4$	0.02M
4)	F-6-P	0.025M
5)	NADH	5.2mM
6)	KCl	0.5M
7)	Dithiothreitol (DTT)	11mM
8)	Enzyme:	

Aldolase	10 mg/ml	) dissolved in 4.7 ml
TIM/αgylcerophosphate	10 mg/ml	) of 0.01M Tris HCl pH 8.0 containing 10 mg B.S.A.

Reaction mixture: Per 10 assays

1)	Tris	2 ml
2)	ATP	1 ml
3)	NADH	1 ml

Figure 2.5 The activity of PFK at different time intervals after homogenisation


µMOLES/G DRY WT TISSUE
40
30
25
50
75
100
125
TIME (MIN)

4) DTT 1 ml

5) KCl 1 ml

6) Enzyme 1 ml

7) H_2O 1 ml

8) $MgSO_4$ 1 ml

Assay:

1) Reaction mixture 1 ml

2) Sample 100 µl (1:10 homogenate dilution)

Start reaction with 100 µl F-6-P.

Because the homogenised solution was particularly unstable with respect to this enzyme (Figure 2.5), PFK activity was measured immediately after the tissue was homogenised.

CYTOCHROME OXIDASE (CYT. OX. E.C.1.9.3.1)
(Cytochrome C:O_2 oxidoreductase)

Cytochrome oxidase is a term which has been used for many years to describe the last carrier or terminal oxidase in the chain of electron transport. It consists of both cytochrome a + a_3; cytochrome a_3 is responsible for the final combination of reducing equivalents with molecular oxygen.

<u>Some of the components of the respiratory chain in mitochondria</u>

Cyt. b → Cyt. c → Cyt. c → Cyt. a → Cyt. a_3 → O_2

<u>Method</u>

To measure the activity of the cytochrome oxidase the rate of oxidation of reduced cytochrome c was followed. A microadaptation of the method of Cooperstein

and Lazarow (1950) was used.

Reagents:

1) Cyt. c 0.03M in phosphate buffer pH 7.4

2) Sodium dithionite 1.2M (fresh daily)

A solution of cytochrome c was completely reduced by addition of sodium dithionite. The solution was shaken vigorously for 2 minutes to remove excess dithionite and a standard cytochrome oxidase preparation was then added to 1 ml of this solution. The rate of decrease in extinction at 550 nm was determined. The original cytochrome c solution was then shaken further and the activity of the same cytochrome oxidase preparation redetermined. This procedure was repeated until further shaking produced no change in the rate of oxidation of the cytochrome c.

Samples were then assayed with the completely reduced cytochrome c solution.

Assay:

Reduced cyt. c	1 ml
Sample	100 µl

Read at 550 nm for 3 minutes.

At the end of the enzymatic reaction a few grains of potassium ferricyanide were added. This completely oxidises the cytochrome c.

The reaction is governed by first-order kinetics; the calculations were therefore carried out as follows:

The optical density of the completely oxidised sample was subtracted from that at any given time and the logarithm of the difference was expressed per minute when

the final tissue dilution was 1:10,000.

Samples to be assayed for cytochrome oxidase activity were kept on ice and assayed within 1 hour of homogenisation. The samples were stable during that period.

β-GLUCURONIDASE (E.C.3.2.31)
(β-D-glucuronide glucuronohydrolase)

β-glucuronidase, a lysosomalacid hydrolase, in skeletal muscle probably participates in the breakdown of sarcoplasmic material (Lockshin and Beaulaton, 1974). Specifically β-glucuronidase cleaves non-reducing terminal β-glucuronosyl residues from glycosamino-glycans and conjugated steroids (Barret and Heath, 1977).

Method

β- glucuronidase was determined using the substrate 4-methylumbelliferyl β-D-glucoronide (MUG) (Robins, Hirsch and Emmons, 1968).

Reagents:

1)	Acetate buffer	0.1M pH 3.5
2)	MUG	1.5mM
3)	Na_2CO_3	1M

Assay: Incubate at 37°C for 60 minutes.

1)	Buffer	0.4 ml
2)	MUG	0.25 ml
3)	Sample	0.1 ml

Stop reaction with 1.5 ml Na_2CO_3.

Free 4-methylumbelliferone was estimated with excitation at 360 nm emission at 448 nm.

Blanks were prepared with each assay, by

incubating the substrate and buffer alone; the enzyme was only introduced after the addition of Na_2CO_3.

The absolute yield of free aglycone was determined by comparing the fluorescence measured in the enzyme assay with a standard curve. The regression line of the free 4-methylumbelliferone against fluorescence was y = 353.2x + 42.2 and was linear over the range 0-0.5nM free 4-methylumbelliferone (r = 0.996).

The assay started not more than 45 minutes after the first sample was homogenised. During this time the enzyme's activity did not alter.

GLYCOGEN

In the horse, glycogen is found widely distributed between fibrils throughout muscle (Figure 2.6) and is an important metabolic fuel.

Glycogen is usually determined by hydrolysis to glucose which is then estimated chemically. The specific method of hydrolysis used in this study was based on that of Huijing (1970) which allows the use of small tissue samples.

<u>Method</u>

The samples were homogenised in de-ionised water to give a mg/ml solution. The hydrolysis of the tissue glycogen involves:

Reagents:

1) Buffer 100 mM acetate buffer pH 4.8
2) Enzyme solution 0.0175 amiloglucosidase(2.45 U/ml).

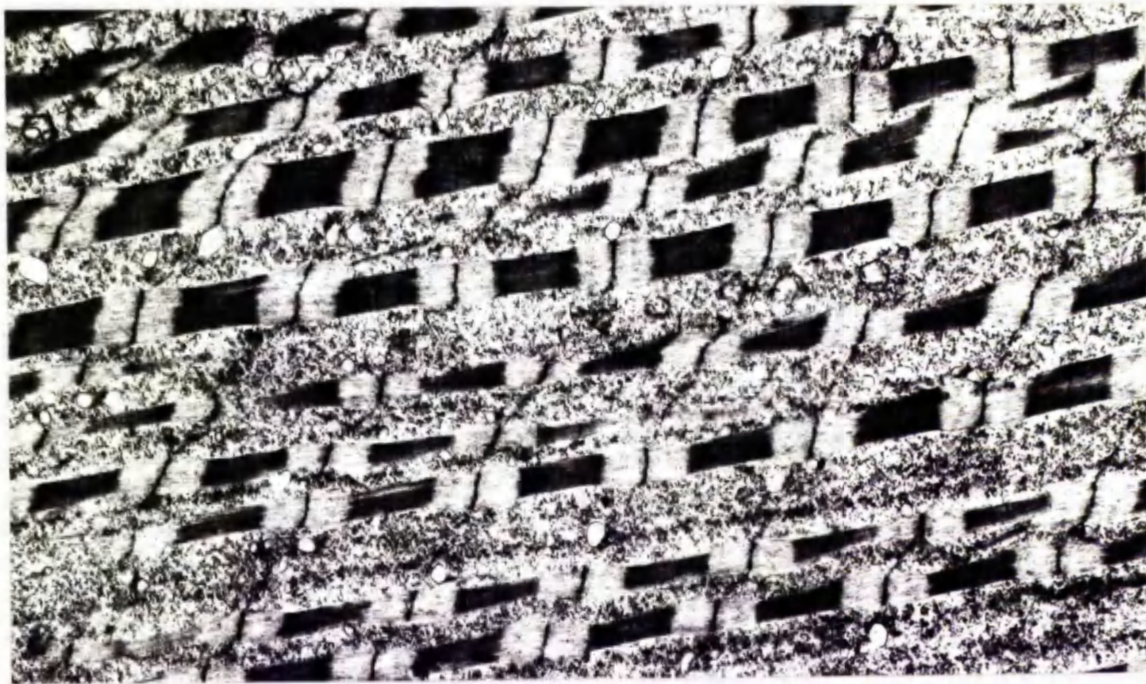


Figure 2.6 Glycogen appears as dark granules between the fibres of horse skeletal muscle

Fixative : Osmium

Buffer : s-collidine

Mag. : 8.4×10^3

and 0.004 ml αamylase (20 U/ml)

1 ml buffer.

Assay:

Enzyme solution	20 µl	
Sample	100 µl	Incubate 60 minutes at 37°C
H_2O	100 µl	

The amount of glucose produced was measured by using a Boehringer No. 124028 test kit for blood glucose.

Method

Reagents:

1) Colour agent - Phosphate buffer pH 7.0 100mM
   Glucose oxidase (> 10 U/ml)
   Peroxidase (> 0.8 U/ml)
   ABTS mg/ml

2) HCl 5N

Assay:

Colour reagent added to sample 0.5 ml

Incubate for 15 minutes at 37°C.

Stop reaction with 0.5 ml HCl.

Read extinction at 436 nm.

Blanks and standards were assayed at the same time and the samples were run in triplicate. The glycogen content was calculated from a standard curve of which the regression line was $y = 0.048x + 0.03$ and was linear over the range 0-900 µmoles glycogen (glucose units) ($r = 0.999$).

TOTAL PROTEIN

Total protein was assayed according to the method of Lowry, Rosebrough, Farr and Randall (1951). The

principle of this assay is based on a coloured complex forming between the tyrosine and tryptophan residues of the protein and an added copper-phenol reagent.

Method

The samples were homogenised in de-ionised water to give a mg/ml solution and then diluted 1:10 with distilled H_2O.

Reagents

1) Na_2CO_3 4% in 0.1M NaOH
2) $CuSO_4$ 0.5%
3) K/Na tartrate 1.0%
4) Folin phenol 0.1M

Reaction mixture:

1) Na_2CO_3 50 ml
2) $CuSO_4$ 1 ml
3) K/Na tartrate 1 ml

Assay:

Reaction mixture 1 ml
Sample/standard 1 ml
Incubate at room temperature for 10 minutes.
Add 0.1 ml folin phenol.
Incubate at room temperature for 30 minutes.
Read at 750 nm on a Beckman spectrophotometer.

The total protein content of the samples was calculated from a standard curve assayed at the same time using horse serum albumin.

The regression line of the standard curve was $y = 0.0043x + 0.06$ and was linear over the range 0-100 µg protein ($r = 0.994$).

The within assay variation was 1.3% and between assay variation was 2.6%.

LACTATE

Tissue handling was extremely important in the samples to be considered for lactate and pyruvate estimations. All samples were powdered under liquid nitrogen; care was taken to remove any contaminating blood or connective tissue from the sample. 3-6 mg of muscle powder was weighed into small tubes and soluble metabolites were extracted by addition of cold 0.5N perchloric acid containing 1 mmol/l EDTA. Perchloric acid was added at a rate of 1 ml per 10 mg muscle powder. This solution was agitated in an ice bath at $-10^{o}C$ for 15 minutes. The solution was centrifuged at $-10^{o}C$ and the supernatant removed. Lactate and pyruvate estimations were not altered by the addition of potassium bicarbonate and therefore this stage was omitted from the procedure outlined by Harris, Hultman and Nordesjo (1974).

Rigorous cleaning of glassware was necessary to gain the necessary precision of the assay. Cleaning of the tubes and glassware was carried out as recommended by Lowry and Passonneau (1972) with certain modifications. The procedure was as follows:

1) Rinse twice in water.
2) Spin upside down and drain through inserted steel insert (Lowry and Passonneau, 1972, p. 67)
3) Fill the tubes with 0.1N NaOH and sonicate for 15 minutes.
4) Centrifuge as 2.

5) Rinse twice with glass-distilled water, centrifuging between.

6) Fill the tubes with 50% HNO_3 and sonicate for 30 minutes.

7) Rinse twice with glass-distilled water. Spin and drain through inserted steel insert.

8) Dry at room temperature.

The concentration of lactate was measured by a modification of the method of Olsen (1972) and was determined at 340/460 nm on a Perkin-Elmer 1,000M fluorimeter.

Method

Reagents:

1)	Buffer - hydrazine	1.1M pH 9.0
2)	Cofactor - NAD crystalline	
3)	Enzyme - lactate dehydrogenase (LDH)	5 mg/ml
4)	Stock lactate solution	5mM

Standards:

Stock solution	(ml)	0	0.1	0.3	0.5	0.7	0.8
Diluent (0.5N $HClO_4$)	(ml)	5.0	4.9	4.7	4.5	4.3	4.2
Lactate	(mM)	0	0.1	0.3	0.5	0.7	0.8

Reaction mixture: Per ml hydrazine buffer

1) NAD 2 mg

2) LDH 10 µl

Assay:

Sample/standard 10 µl

Reaction mixture 100 µl

Incubate 60 minutes at 25°C.

Add 1 ml of H_2O

Read fluorescence at 340/460 nm.

The regression line for the standard curve was y = 372.1x + 162.7 (r = 0.999). The response was linear over the range 0-4.0 mmol/l lactate. All samples and standards were carried out in duplicate. Within assay variation was 2.1% and between assays was 2.5%.

PYRUVATE

Samples and glassware were handled in a similar fashion to the lactate assay.

Method (Olsen 1972)

Reagents:

1) Buffer - phosphate 1M pH 7.0
2) Cofactor - NADH 2mM
3) Enzyme - LDH 0.01 mg/ml
4) Stock pyruvate solution 2mM

Standards:

Stock solution	(µl)	0	10	10	10
Diluent (0.5N $HClO_4$)	(ml)	2	4	2	1
Pyruvate	(µM)	0	5	10	20

Reaction mixture: Per ml phosphate buffer

1) NADH 6 µl
2) LDH 20 µl

Assay:

Sample standard 50 µl

Reaction mixture 100 µl

Incubate 60 minutes at 25°C.

Add 1 ml carbonate buffer pH 10.0 (20mM).

Read fluorescence at 340/460 nm.

The regression line for the standard curve was $y = 7.78x + 462.4$ ($r = 0.997$). The response was linear over the range 0-0.5 mmol/l pyruvate.

All samples and standards were carried out in triplicate. The within assay variation was 2.9% and between assay variation was 3.2%.

URINE ANALYSES

Twenty-four hour urine samples were collected by means of a collection bag attached to a harness (Weir and Gifford, 1971) which was emptied on several occasions during the 24 hour period. Total urine volume per 24 hours was measured. From each urine collection during the 24 hours an aliquot (1%) was removed and 1M sulphuric acid added to give a 1% solution and kept at 4°C. At the end of the 24 hour period these aliquots were pooled for the determination of urinary nitrogen concentration (automated Kjeldahl-Kjeldahl Electric). An aliquot of the 24 hour pool was also obtained for the measurement of total creatinines (continuous flow analysis - Autoanalyser II, method 2).

SECTION 3

AN INVESTIGATION OF SOME EFFECTS OF AN ANABOLIC STEROID IN THE RESTING HORSE

Information on the effects of anabolic steroids in horses is limited. Studies in general have been conducted on debilitated animals (O'Connor, Stillions, Reynolds, Linkenheimer and Maplesden, 1973) and/or on animals in training (Vigre, 1963; Stihl, 1968; Dietz *et al*, 1973; Dawson and Gersten, 1978). Debilitated animals showed increased weight in conjunction with an improved nitrogen retention when administered 17β-hydroxyandrosta-1,4-dien-3-one, 17-(10-undecenate), (boldenone undecylenate) (O'Connor *et al*, 1973). A similar increase in weight was noted with CIBA 29038-Ba as a result of improved appetite in debilitated racing animals (Stihl, 1968). The effect was greatest in geldings and least in stallions.

Under the rules of racing in Great Britain, horses receiving anabolic steroids cannot race. This legislation does not apply in the U.S.A. From a questionnaire circulated in both countries (Snow and Munro, unpublished) it was possible to gain some information on their use in horses. A response from both countries gave a total of almost 100 replies and from these it was possible to draw the following conclusions.

It was evident that approximately 90% of the practitioners who replied were using anabolic steroids. Their administration was primarily for therapeutic use although about 30% of the practitioners in the States

were using them on horses involved in competition.

In the U.K. the most commonly used drug was nandrolone phenylpropionate. Therapeutic dose rates were being used by 70-80% of the practitioners whilst the others were using lower doses. It was interesting to note that quite a number of practitioners were using testosterone.

The view held by almost all the practitioners in both countries was that anabolic steroids improved performance. They also believed that this was the view held by most owners and trainers.

Before embarking on a study to look at the effects of anabolic steroids on the healthy exercising horse, it was important to establish the effects of the anabolic drug in the resting animal. This section is concerned with that study and looks at a number of relevant parameters associated with the skeletal musculature of healthy mature castrate horses.

MATERIALS AND METHODS

ANIMALS

Four healthy mature geldings (3 Thoroughbred TB1-TB3 and 1 Heavy Hunter HH1) aged between 6 and 13 years were used in this study.

FEED

For the first week of the study the animals were fed on hay; thereafter to minimise the possible effects of dietary variation, the horses were fed exclusively on a constant diet of known composition. A commercial preparation of horse cubes (Spillers "Complete Horse Cubes") was given at the manufacturer's recommended rate of 9.1 Kg/day. The composition of the diet was analysed at 114 g crude protein (CP), 28 g ether extract, 240 g crude fibre/Kg dry matter (DM) (Parkins and Snow pers. comm.). To prevent the possible intake of straw bedding, the horses were bedded on peat.

EXERCISE

During the entire period of this investigation only maintenance exercise, consisting of being walked 1-2 Km 5 days per week was given.

EXPERIMENTAL PROTOCOL

The study comprised three consecutive periods, pre-treatment (PT), treatment (T) and post-treatment (Post T).

TREATMENT

Nandrolone phenylpropionate in oil (Nandrolin,

Intervet) was injected intramuscularly in the neck region. Injections were given at weekly intervals at a dose of 400 mg, corresponding to the recommended upper therapeutic dose (Intervet data sheet). A total of 7 injections was given to each animal.

OBSERVATIONS

Throughout the study any alterations in the animals' temperament and response to the presence of other horses or personnel were noted. A range of blood biochemistry parameters was determined at weekly intervals. Body weights of the horses were also recorded weekly.

URINE

24 hour collections were taken during the PT period on three occasions, over a 16 day period prior to the commencement of treatment. During the T period sampling commenced on the day of the second anabolic injection and continued weekly, with the last sample being taken 1 week after the seventh injection. Post-T samples were then taken on four occasions at weekly intervals.

Urinary nitrogen and total creatinine were measured from these samples (Section 2). From the nitrogen data obtained it was possible to make some speculative estimations of the nitrogen retention of each horse, before, during and after anabolic steroid administration.

In a separate experiment (Parkins and Snow, unpublished), the same diet was given to 4 horses in a carefully controlled digestibility trial. In this experiment the total faecal output of each horse was

collected over a 5 day period using a body harness and faecal bag. The diet contained 114 g CP/Kg DM. The mean digestibility coefficient for the CP fraction was found to be 0.72 ± 0.023. From this it was calculated that the diet contained 82 g digestible crude protein (DCP)/Kg DM. Thus for the experimental animals in the current study that were given 9.1 Kg fresh matter/day (8.1 Kg DM) the calculated mean faecal CP output was 260 g/day (42gN). N retention was calculated by subtracting this assumed daily faecal output of 42gN together with the experimentally determined urinary N output from the N input of the diet.

MUSCLE

Percutaneous needle biopsies were taken and prepared for biochemical and histochemical analyses, as outlined in Section 2, from 2 hind limb muscles, the biceps femoris (BF) and semitendinosus (Semi-T). Biopsies were obtained on four occasions over an 8 week period prior to the commencement of treatment. In the T period samples were obtained, 1, 4 and 6 weeks after the first injection and in the post-T period, 2 weeks after the last injection.

Histochemical samples were stained for myosin ATP-ase pH 9.4 with preincubation at pH 10.2 and succinate dehydrogenase (Section 2) and subsequently divided into 3 fibre types, FT, FTH and ST.

From the freeze-dried specimens, water content, total protein concentration, glycogen concentration and

the activities of 6 muscle enzymes, aspartate amino-
transferase (AST), alanine aminotransferase (ALT),
citrate synthase (CS), lactate dehydrogenase (LDH),
aldolase (ALD) and 3-hydroxyacyl-CoA dehydrogenase (HAD)
were determined.

RESULTS

GENERAL

Shortly after the change in diet to the complete cube ration, coprophagia was observed to varying degrees in all the horses. In one animal, HH1, a marked increase in water consumption was noted. Behavioural changes occurred after nandrolone administration. After the second or third injection it was found that the geldings gradually exhibited some components of stallion behaviour e.g. flehmen, increased aggressiveness to other geldings and an increased tendency to bite (Figure 3.1). About 2 weeks after the last anabolic injection the horses had returned to their normal relatively quiescent characteristics. Although the blood biochemistry results showed some statistical alterations throughout the experimental period none was outwith the normal clinical range (Department of Veterinary Clinical Biochemistry - unpublished) (Table 3.1).

The average body weights in the pre-treatment period in the individual horses were - TB1:481 ± 5.0 Kg; TB2:530 ± 4.8 Kg; TB3:540 ± 0.6 Kg; and HH1:692 ± 7.8 Kg. Throughout the periods of treatment and post-treatment the body weights of the individual horses remained within 1% of the average pre-treatment values.

URINE

The 24 hour urine volumes for the individual horses over the complete period of study are illustrated in Figure 3.2. A marked transient increase in urine

Figure 3.1 Androgenic effects of anabolic steroids

TABLE 3.1 Effect of nandrolone on blood biochemistry
(Mean ± S.E.M. of mean from 4 horses)

		PT (3)	T (7)	Post-T (4)
Total protein	g/l	66 ± 2.4	69 ± 1.9	70 ± 1.8
Urea	mmol/l	5.9 ± 0.49	5.8 ± 0.36	6.6 ± 0.31***
Creatinine	μmol/l	179 ± 7.7	157 ± 15.8	163 ± 8.1
Glucose	mmol/l	4.9 ± 0.23	4.9 ± 0.10	4.8 ± 0.11
Cholesterol	mmol/l	1.84 ± 0.075	2.06 ± 0.133	+++2.53 ± 0.154***
PCV	l/l	0.38 ± 0.028	0.36 ± 0.019	0.39 ± 0.024
Sodium	mmol/l	141 ± 1.5	141 ± 0.8	139 ± 1.3
Potassium	mmol/l	3.6 ± 0.04	3.6 ± 0.18	3.7 ± 0.13
Chloride	mmol/l	97 ± 0.2	100 ± 0.6	99 ± 0.5
Calcium	mmol/l	2.89 ± 0.069	2.79 ± 0.022	2.75 ± 0.054
Magnesium	mmol/l	0.66 ± 0.028	0.62 ± 0.014	0.58 ± 0.010*
Phosphate	mmol/l	1.45 ± 0.121	1.17 ± 0.044	1.35 ± 0.028
Bilirubin	μmol/l	32 ± 2.5	31 ± 2.7	34 ± 3.6
Alk. Phos	I.U.	88 ± 8.8	82 ± 10.4	106 ± 11.1
AST	I.U.	253 ± 16.8	250 ± 11.6	241 ± 17.2
γGT	I.U.	11 ± 1.2	17 ± 1.9†	+15 ± 1.0

Significance tests:

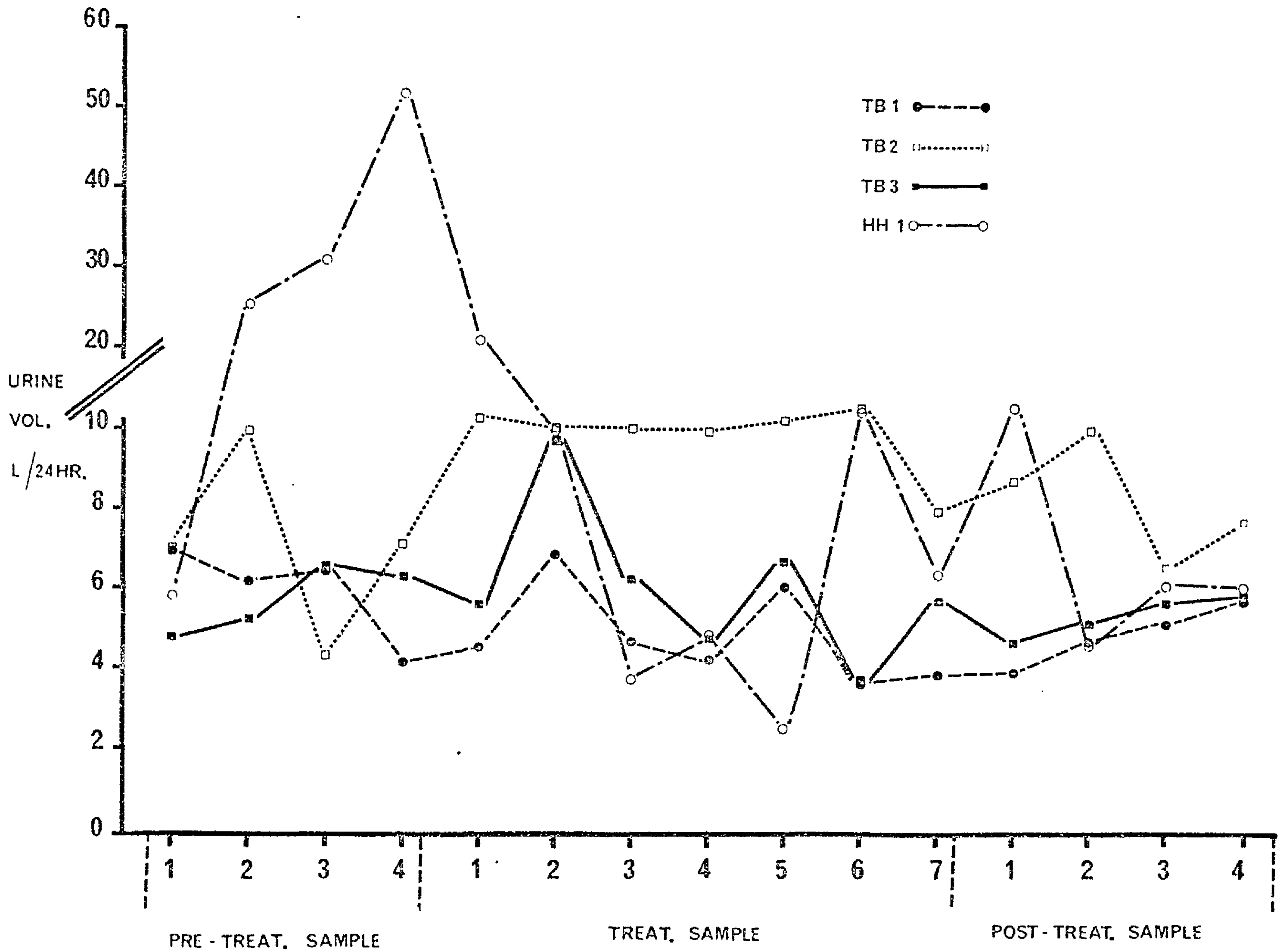
T versus Post-T * $p < .05$ *** $p < 0.001$

PT versus Post-T + $p < .05$ +++ $p < 0.01$

PT versus T † $p < 0.05$

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

Figure 3.2 Urine volume (L/24 hrs.) throughout the period of investigation


TB 1
TB 2
TB 3
HH 1
URINE
VOL.
L/24 HR.
60
50
40
30
20
10
8
6
4
2
0
1 2 3 4 1 2 3 4 5 6 7 1 2 3 4
PRE - TREAT. SAMPLE
TREAT. SAMPLE
POST - TREAT. SAMPLE

volume of horse HH1 was observed in the initial period of the investigation. The mean nitrogen excretion (mg/Kg/day) and estimated nitrogen retention values (mg/Kg/day) for each horse in each period is shown in Table 3.2. Nitrogen excretion decreased significantly ($p < .05$) in 3 animals during the treatment period, with a return towards PT values during the Post-T period. However when expressed in terms of nitrogen retention, the change was significant in only 2 horses. Creatinine excretion in individual horses for all periods is shown in Table 3.3.

MUSCLE

No difference was found, in any of the muscle parameters measured, between the first biopsy, taken while the animals were still on hay, and the other 3 biopsies of the PT period. The 4 values have therefore been averaged for the 4 horses to give a mean control value for the PT period.

HISTOCHEMISTRY

Table 3.4 gives the mean results of histochemical division of the muscle fibres into their 3 types. A general observation is that the BF has a higher percentage of ST fibres than the Semi-T ($p < 0.02$).

The percentage of each fibre type did not alter significantly in the Semi-T, throughout the period investigated. However in the BF, there was a significant increase in the proportion of fast twitch low oxidative fibres and a concomitant decrease in the fast twitch high oxidative fibres, during treatment. This occurred in

TABLE 3.2 Effect of nandrolone on urinary nitrogen excretion and estimated nitrogen retention (Mean ± S.E.M.)

Horse No.	Nitrogen excretion (mg/Kg body wt/day)			Nitrogen retention (mg/Kg body wt/day)		
	PT (3)	T (7)	Post-T (4)	PT (3)	T (7)	Post-T (4)
TB 1	192.3 ± 5.4	158.2 ± 12.0*	182.0 ± 13.4	13.4 ± 2.6	29.9 ± 5.8	19.1 ± 5.4
TB 2	167.0 ± 27.9	201.5 ± 8.6	212.4 ± 10.4	18.8 ± 14.5	0 ± 11.9	-5.0 ± 5.5
TB 3	173.5 ± 12.3	133.7 ± 10.8*	160.6 ± 9.9	12.1 ± 6.7	33.7 ± 5.9*	18.4 ± 6.5
HH 1	186.1 ± 20.6	102.4 ± 17.1	119.1 ± 9.3	-23.2 ± 14.4	28.6 ± 11.9*	23.4 ± 6.5

* = $p < 0.05$ compared to pretreatment values

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

TABLE 3.3 Effect of nandrolone on urinary creatinine excretion (Mean ± S.E.M.)

Horse No.	Creatinine excretion (µmole/Kg body wt/day) PT (3)	T (7)	Post-T (4)
TB1	251 ± 13.5	290 ± 16.6	291 ± 13.0
TB2	206 ± 26.3	323 ± 14.1***	312 ± 13.9**
TB3	247 ± 38.6	249 ± 21.4	250 ± 17.7
HH1	276 ± 26.6	227 ± 27.6	218 ± 9.1

** = $p < 0.02$ compared to pretreatment values

*** = $p < 0.01$ compared to pretreatment values

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

TABLE 3.4 Effect of nandrolone on percentage fibre composition of 2 hind limb muscles (Mean ± S.E.M. n = 4 horses)

Muscle	Classification	PT (4)	T		Post T
			4 wk	6 wk	2 wk
B.F.	Fast twitch low oxidative	25.3 ± 2.5	36.6 ± 1.2***	33.7 ± 1.6*	32.5 ± 0.4
Semi-T	" "	36.2 ± 2.6	43.1 ± 2.2	37.9 ± 3.6	37.4 ± 1.9
B.F.	Fast twitch high oxidative	58.8 ± 1.6	44.8 ± 2.4***	47.3 ± 1.7**	50.2 ± 3.4*
Semi-T	" "	59.9 ± 2.0	48.1 ± 3.1	54.6 ± 4.0	58.5 ± 3.4
B.F.	Slow twitch high oxidative	15.9 ± 3.4	18.6 ± 2.6	19.0 ± 1.6	17.3 ± 3.1
Semi-T	" "	3.9 ± 0.8	8.8 ± 2.2	7.5 ± 1.2	4.1 ± 3.7

* = $p < 0.05$ compared to pretreatment values B.F. = biceps femoris

** = $p < 0.02$ " " " Semi-T = semitendinosus

*** = $p < 0.01$ " " "

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

all 4 animals.

BIOCHEMISTRY

The water content of the muscle samples did not alter throughout the entire period of the investigation, nor did the total protein content (Table 3.5). The inter-animal variation in glycogen content was quite marked although in all cases the Semi-T had significantly less glycogen ($p < 0.01$) than the BF (Table 3.5). The glycogen content of neither muscle was, however, affected by nandrolone phenylpropionate administration. The activities of the range of muscle enzymes determined showed no significant alteration with anabolic steroid administration (Table 3.6).

TABLE 3.5 Effect of nandrolone on the percentage water content, total protein and glycogen concentration of 2 hind limb muscles (Mean ± S.E.M. n = 4 horses)

	Muscle	PT	T			Post-T
			1 wk	4 wk	6 wk	2 wk
% Water	B.F.	(3) 72.8 ± 0.2	72.7 ± 0.3	72.9 ± 0.9	73.3 ± 1.0	72.4 ± 0.5
	Semi-T	72.1 ± 0.3	73.2 ± 0.3	73.5 ± 0.6	73.9 ± 0.6	73.1 ± 0.8
Total protein mg/g dry wt	B.F.	(4) 575.0 ± 33.5	527.5 ± 37.0	565.0 ± 37.5	612.5 ± 47.5	567.5 ± 16.0
	Semi-T	561.7 ± 27.5	522.5 ± 41.5	527.5 ± 48.0	550.0 ± 29.7	575.0 ± 26.3
Glycogen µmole/g dry wt	B.F.	(4) 535.7 ± 73.9	572.7 ± 83.6	517.2 ± 73.3	488.2 ± 62.7	485.0 ± 61.8
	Semi-T	444.5 ± 57.1**	418.7 ± 74.6**	415.7 ± 57.1**	400.0 ± 56.1**	356.2 ± 42.6**

** Significant difference at $p < 0.02$ between BF v Semi-T B.F. = biceps femoris

Semi-T = semitendinosus

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

TABLE 3.6 Effect of nandrolone on the activity of several muscle enzymes (μmole/min. g dry wt) of 2 hind limb muscles (Mean ± S.E.M. n = 4 horses except LDH where n = 3 horses)

Enzyme	Muscle	PT (4)	T			Post-T
			1 wk	4 wk	6 wk	2 wk
AST	B.F.	560 ± 87	496 ± 11	472 ± 25	499 ± 34	505 ± 34
	Semi-T	480 ± 65	435 ± 41	452 ± 36	389 ± 39	352 ± 55
ALT	B.F.	50.4 ± 6.5	50.4 ± 5.2	49.7 ± 6.5	47.4 ± 3.7	48.3 ± 5.0
	Semi-T	46.8 ± 3.4	45.9 ± 1.6	44.2 ± 4.4	40.6 ± 3.0	39.8 ± 2.2
CS	B.F.	16.9 ± 0.7	18.6 ± 2.6	17.9 ± 1.9	17.6 ± 2.1	17.8 ± 1.7
	Semi-T	11.8 ± 0.6	11.1 ± 1.3	13.6 ± 0.9	12.2 ± 0.8	12.4 ± 0.8
LDH	B.F.	2200 ± 80	2250 ± 40	2260 ± 90	2110 ± 40	2380 ± 30
	Semi-T	2520 ± 180	2970 ± 270	2920 ± 390	2210 ± 150	2470 ± 90
ALD	B.F.	211 ± 9	208 ± 16	213 ± 10	221 ± 18	220 ± 22
	Semi-T	256 ± 22	249 ± 23	300 ± 26	256 ± 19	244 ± 16
HAD	B.F.	117 ± 19	110 ± 16	116 ± 19	130 ± 15	143 ± 10
	Semi-T	107 ± 19	114 ± 16	113 ± 10	112 ± 13	113 ± 13

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

B.F. = biceps femoris
Semi-T = semitendinosus

DISCUSSION

The mild stallion behaviour is attributable to a residual androgenic activity in the compound used. The coprophagia seen in all animals is a fairly common finding in horses (Slade, Robinson and Casey, 1970), and in this instance may have been accentuated by the managerial practices necessitated by the experiment. Similarly in the absence of any clinical cause of the condition, the large transient increase in urine volume of one of the horses (HH1) is considered a result of polydypsia that in turn reflected the animal's boredom with the diet and lack of exercise. With the above exception, the urinary production observed was at the lower limits of that previously reported in both ponies (Rawlings and Bisgard, 1975), and larger breeds (Gelsa, 1979).

In this study anabolic steroid-induced effects on skeletal muscle have been sought by measuring several parameters. The study indicates that in the castrated mature horse nandrolone at the dosage used (approximately 1 mg/Kg) does not promote an increase in body weight. This contrasts with the findings of O'Connor *et al* (1973) who reported variable weight gains with boldenone undecylenate. It may be that the differing responses of the animals in the two studies reflects the differing body conditions of the animals at the onset of treatment. Compared to the mature healthy animals used in the current investigation, those studied by O'Connor *et al* (1973)

were debilitated. Although there is extensive literature on the actions of anabolic agents on body weight in other species there are no reports which examine the effects of an anabolic steroid on healthy mature castrates. Increases in body weight of healthy rats receiving the anabolic steroid methylandrostenediol (MAD) have been reported but these animals were immature (Korner and Young, 1955). Reports of body weight increases in castrated adult guinea-pig (Kochakian, 1949) and castrate adult monkeys (Phoenix, 1974) have used testosterone as the anabolic agent. When an anabolic steroid was administered to healthy mature rats (Hickson *et al*, 1976a) and healthy mature rabbits (Salmons in press) no body weight increases were noted. However these latter studies were carried out on intact males and females respectively.

Urinary nitrogen excretion was used as an indication of anabolism (Kochakian, 1935; Kochakian and Murlin, 1935; Chan, Heitzman and Kitchenham, 1975). The significantly increased nitrogen retention in 2 animals in this study does suggest an anabolic action. However when these changes are calculated out on the basis of protein gain over the period of treatment, only an approximately 1% increase in body weight would be expected. An increase of this magnitude would not be detected under the experimental conditions.

The mean levels of urinary creatinine are similar to those reported by Rawlings and Bisgard (1975) in the pony and in mixed breed mares (Traver, Salem, Coffman,

Garner, Moore, Johnson, Tritschler and Amend, 1977). The skeletal muscle mass of large animals can be assessed by measuring the creatinine output in the urine (Van Niekerk, Reid, Bensadoun and Paladines, 1963) if the diet is kept constant (Wood, Schneeman, Zezulka, Calloway and Margen, 1976) and excretion is measured over a sufficiently long period (Peters, 1973). However, creatinine is a product of muscle breakdown and if the degradation of muscle increases in both anabolic and catabolic states (Millward *et al*, 1978) then this interpretation of creatinine excretion may only be used in steady state conditions. The findings in the present study of unaltered creatinine excretion in those animals where nitrogen retention occurred is in agreement with the reports in man of unaltered creatinine excretion during testosterone propionate treatment of an eunuchoid (Landau, Knowlton, Lugibihl, Brandt and Kenyon, 1950). In TB2 the increased urinary creatinine excretion cannot be readily explained.

The investigation into the composition of the skeletal muscle of the hind limbs of the animals in the present study was considered to be of particular relevance to the subsequent investigation on the interrelation of exercise and anabolic steroid administration. Few studies have been carried out on the growth-promoting effects of anabolic steroids on limb muscles. The more responsive temporal muscle of the female guineapig has shown changes in fibre type and area (Cobban and Salmons, pers. comm.) and a decrease in oxidative capacity (Gutmann and

Hanzlikova, 1970; Cobban and Salmons, pers. comm.) when subjected to anabolic agents. Although these changes have not been reported in limb muscles there are reports of increased water (Casner *et al*, 1971) and glycogen content (Bergamini, 1975) in skeletal muscles on administration of anabolic agents.

The proportion of FT and ST fibres in this study was unaltered by nandrolone administration. This is in contrast to Cobban and Salmons (pers. comm.) findings where there was a decrease in the number of alkali labile (ST) fibres with nandrolone administration. The discrepancy is most likely explained by the different muscle studied.

A change in the percentage of FT and FTH fibres was noted in the BF but not the semi-T in this study. A variable response of different muscles to androgenic steroids is not a new phenomenon. Kochakian and Tillotson (1957) noted this effect in a detailed study of the growth response of a number of skeletal muscles of the guineapig. The decrease in the percentage of FTH fibres in the BF is similar to an effect noted in the temporal muscle of the female guineapig when treated with testosterone post-natally (Gutmann and Hanzlikova, 1970). The female temporal muscle is 'red' and high in oxidative capacity. This enzyme pattern was converted to the male 'white' and low oxidative type by daily injection of 40 mg/Kg testosterone propionate to one-month old animals for a period of 1½ months. A similar response has been noted in the temporal muscle of the adult female guineapig with nandrolone phenylpropionate

administration (Cobban and Salmons, pers. comm.). In addition to the change in oxidative capacity found in both these studies, there was a marked hypertrophy of the temporal muscle. Although no fibre area measurements were carried out in the present study it is possible that hypertrophy was occurring. Such an increase in fibre size may not be accompanied by increases in mitochondria (Goldspink and Waterson, 1971) which would cause a dilution effect of the succinate dehydrogenase and a classification of a previously high oxidative fibre as low oxidative.

Increases noted in muscle mass with anabolic steroid treatment have been attributed to an increase in the water content of the tissue (Casner *et al*, 1971). However, in the present investigation and in that of Hickson *et al* (1976a) the water content of the muscles remained unchanged.

The study by Hickson *et al* (1976a) also agrees with the present findings that there was no alteration in the glycogen concentration of the limb muscles. A number of studies administering testosterone to rats have reported increases in both the sex-dependent and limb muscles (Leonard, 1952; Meyer and Hershberger, 1957; Bergamini, 1975) although the mechanism of response in the two types of muscles differs.

Total protein content did not alter in the present study throughout the period of investigation. Similarly Korner (1955) found that injections of MAD to

rats caused no significant change in the myofibrillar, sarcoplasmic or collagen fractions of several muscles.

Reports of changes in enzyme activity with anabolic steroid administration are not so clearly documented. The enzyme citrate synthase was measured in this study to represent the activity of the tricarboxylic acid (TCA) cycle and it did not alter with anabolic steroid administration. The activity of other TCA enzymes, in particular SDH, did not change in skeletal muscle of castrate rats treated with testosterone (Leonard, 1950) although Loring, Spencer and Vilee (1961) did find slight increases in this enzyme's activity as well as increases in malate dehydrogenase and citrate dehydrogenase. However, the latter group included the highly responsive perineal complex as part of a group of muscles used as the homogenate for the enzyme assays. A detailed study carried out on rats treated with nandrolone decanoate (Exner *et al*, 1973) found no changes in the activity of the TCA enzymes. In general, therefore, the present findings of no change in the activity of CS are in agreement with the already published literature. The absence of any decrease in CS activity does not contradict the histochemical findings of a decrease in the percentage of FTH fibres, as the enzyme activity is measured per milligram of tissue. This gives no clue as to the number of fibres within that milligram or of the distribution of the enzyme throughout the tissue. These facts could only be resolved by single fibre analysis.

The influence of testosterone on the glycolytic enzymes aldolase and lactate dehydrogenase have been investigated in the BF muscle of castrated rats (Apostaklis, Matzelt and Voigt, 1963). No changes were reported in the LDH activity. After an increase in aldolase activity with castration, levels returned to those of controls after testosterone treatment. However, these changes were small and questionable (Kochakian, 1976). Support of the present findings where no alteration was found in LDH activity is again provided by Exner *et al* (1973) although no further studies have investigated the effects of anabolic steroids on the activity of aldolase.

The transaminase activity of alanine amino-transferase and aspartate aminotransferase suggested the possible role of these enzymes in a protein anabolic action of androgens. However, the lack of any change in their activity with nandrolone administration is similar to the finding of Kochakian and Endahl (1957) who found no alteration in the transaminases when castrated rats were injected with testosterone.

HAD activity is indicative of the oxidation of fatty acids. Although there are reports of a change in fat metabolism with anabolic steroid administration (Korner and Young, 1955; Heitzman, 1976) no change in the activity of this enzyme was noted.

In general, the lack of any change in enzyme activities would support the conclusions of Exner *et al* (1973) who found no change in a number of enzyme activities of the rectus femoris muscle, including LDH, HAD and SDH

in resting intact male rats treated with nandrolone decanoate. In contrast, they found increases in most enzyme activities in resting female rats treated similarly. The magnitude of the increase was comparable to exercised controls.

SECTION 4

THE EFFECT OF AN ANABOLIC STEROID ON THE SKELETAL MUSCLE OF THOROUGHBRED HORSES DURING A TRAINING AND DETRAINING PERIOD

Detailed studies have been carried out on the effects of training on the skeletal muscle of both Standardbred (Lindholm and Piehl, 1974) and Thoroughbred horses (Straub, Howald, Gerber, Piehl and Pauli, 1975; Guy and Snow, 1977a, b; Snow and Guy, 1979). These authors found an improvement in the aerobic capacity and glycogen content of skeletal muscle of trained animals. Guy and Snow (1977a) also found an increase in the glycolytic enzymes with training.

A variety of training régimes has been used in an attempt to improve an animal's racing performance. In the study by Guy and Snow (1977a, b) a form of interval training was used in which days of endurance training were interspersed with days of repeated sprinting. As skeletal muscle adaptation is specific to the training stimulus (Costill *et al*, 1979) it was of interest to extend the work of Guy and Snow (1977) and investigate the effects of a different training programme on the skeletal muscle of Thoroughbred horses. The training programme selected for the present study involved an initial period of endurance work followed by a period of isolated maximal gallops.

In conjunction with the above training programme, the effects of nandrolone phenylpropionate administration were investigated. In debilitated horses anabolic steroids have been shown to improve racing performance (Vigre, 1963; Stihl, 1968). The greatest improvement was noted in

geldings and was most pronounced over the longer distances (Stihl, 1968). Only one report (Dietz *et al*, 1974), however, has looked objectively at the effects of an anabolic steroid in healthy racing horses. Muscle groups important in movement were examined. The authors found increases in the muscle masses which were specifically involved in the movement, that is, in trotting horses, they found increases in the rump and hind limbs whereas in galloping horses the greatest increases were in the chest and neck. However the results were not consistent and adequate controls were not included in the study.

This present investigation was designed to study the effects of a particular training programme on biochemical and morphological parameters of skeletal muscle. In addition,the effects of nandrolone phenylpropionate on that protocol were investigated.

MATERIALS AND METHODS

HORSES

Six clinically normal gelding Thoroughbreds (Nos. 1, 2, 3, 5, 6, 7) were used.

FEEDING

As in the resting study, the dietary intake was 9.1 Kg/day. On advice from a nutritionist the recommended maintenance diet was augmented 1 week after the onset of the training programme with a protein supplement of 1 Kg/day of Spillers "Concentrate"(3.0% oil, 26.0% protein, 8.5% fibre). Addition of concentrate was terminated following the first urine collection after cessation of training. The horses were bedded on peat.

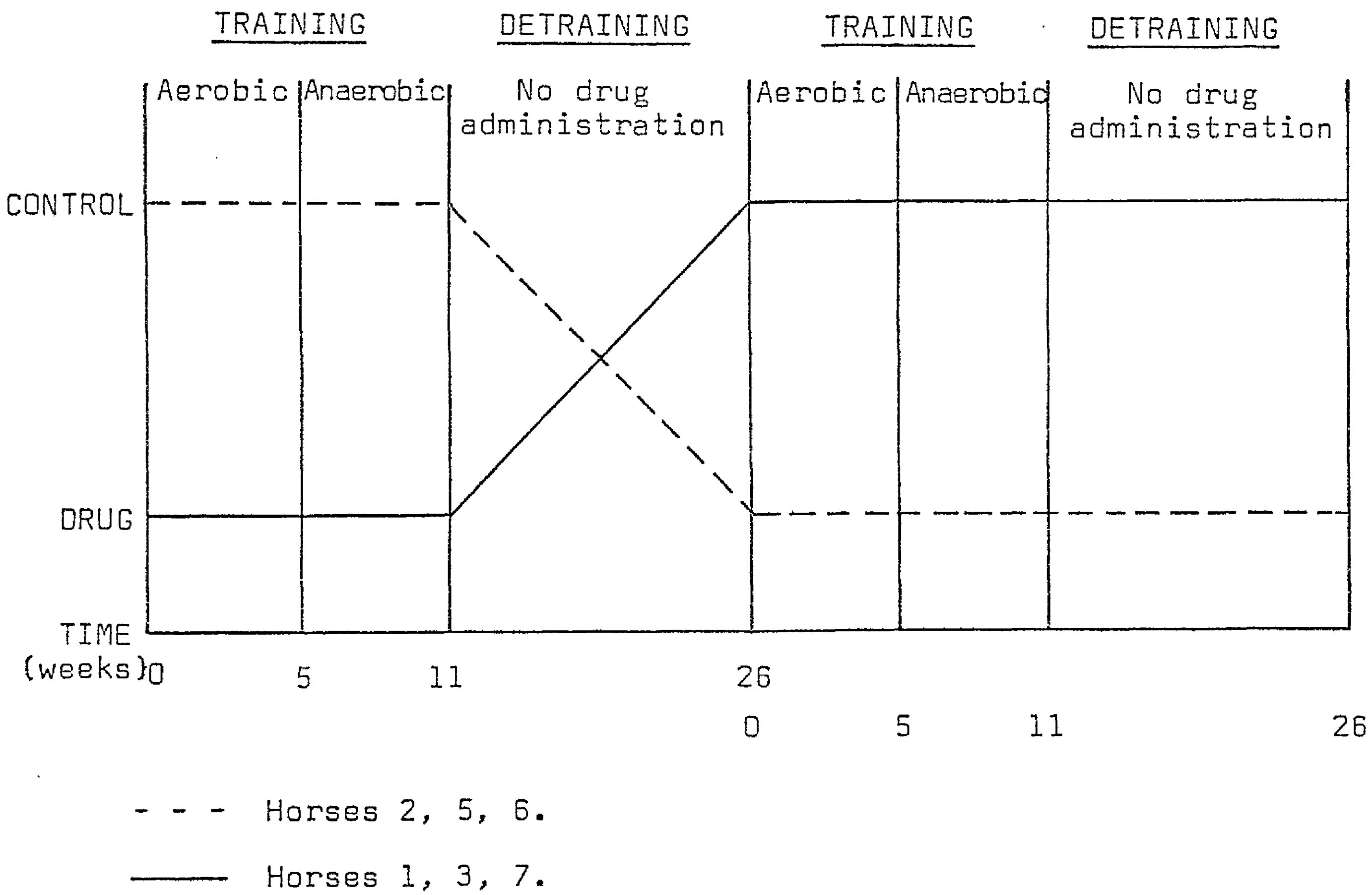
ANABOLIC STEROID

The study was conducted on a cross-over basis (Figure 4.1). The horses were divided into 2 equal groups. The treated group was given 400 mg nandrolone phenylpropionate intramuscularly at weekly intervals during the training period. The control group was simultaneously given an arachis oil placebo. Those horses acting as controls in the first part of the cross-over (cross-over 1) were numbers 2, 5 and 6. At the end of the first part, the control animals acted as treated for the second part of the cross-over.

TRAINING

The training consisted initially of 5 weeks of aerobic/endurance work in an indoor riding school. The horses were progressively trained until they were trotting and cantering for 40 minutes 5 days per week. This period

FIGURE 4.1 Diagrammatic representation of the training and drug administration protocol



of training was followed by 6 weeks of anaerobic/ sprint training during which time the horses were housed and exercised at a racecourse. The anaerobic component of the training consisted of gallops over 506m (approx. 3 furlongs), 1025m (approx. 5 furlongs) and 1600m (approx. 8 furlongs) weekly interspersed with a day of trotting and cantering for approximately 20 minutes. At the end of the 6 week period some of the horses were galloped over 3620m (approx. 18 furlongs). Following this, the horses were returned to the Veterinary School for a 15 week detraining period in which their only exercise was being led at the walk. The horses were then 'crossed-over' and the cycle repeated. Some of the training gallops were selected to act as experimental trials in which performance was assessed and muscle and blood samples were obtained. During these experimental trials each animal ran individually and was ridden by the same jockey.

BODY WEIGHT AND BODY MEASUREMENT

The pretraining body weights were recorded on 3 occasions over a period of 3 weeks prior to the onset of training and are presented in the text as the mean ± S.E.M. of n horses. The 13, 14, 15 week detraining samples of cross-over 1 acted as the pretraining sample for cross-over 2. During aerobic training, weights were recorded at 1, 2, 3, and 5 weeks. Due to the lack of facilities, it was not possible to weigh the horses during the anaerobic period at the racecourse. However during detraining the weights were recorded weekly.

In addition to weighing, body measurements were taken at 5 constant reference points. They were chest, upper fore limb, girth, flank and stifle to stifle (Figure 4.2). The measurements were taken at approximately 2 weekly intervals. The pretraining values were recorded at similar time intervals to the body weight measurements.

URINE SAMPLES

Twenty-four hour urine samples were collected weekly and analysed for total nitrogen and creatinine content (Section 2). The 13, 14, and 15 week detraining samples of cross-over 1 acted as -2, -1, 0 weeks samples of cross-over 2.

MUSCLE BIOPSY SAMPLES

The needle biopsy technique was used (Section 2) to obtain samples from 3 muscles, the middle gluteal (MG), BF and Semi-T. The pretraining (PT) biopsy samples of cross-over 1 were taken just prior to the onset of training. In cross-over 2 the PT sample was obtained 15 weeks following the previous training period. Biopsy samples were then taken at the end of the aerobic training period (post-aerobic) and at the end of the anaerobic training period (post-anaerobic) and then at 2, 4, 6, 9 and 12 weeks following training (detraining).

As the frequency of biopsying increased during the detraining period tranquillisation was necessary in most animals. Azaperone ("suicalm" 4% w/v Crown Chemical Co. Ltd.) was administered intramuscularly at a dose rate of 0.7 mg/Kg^{-1} as described by Mackenzie and Snow (1977).

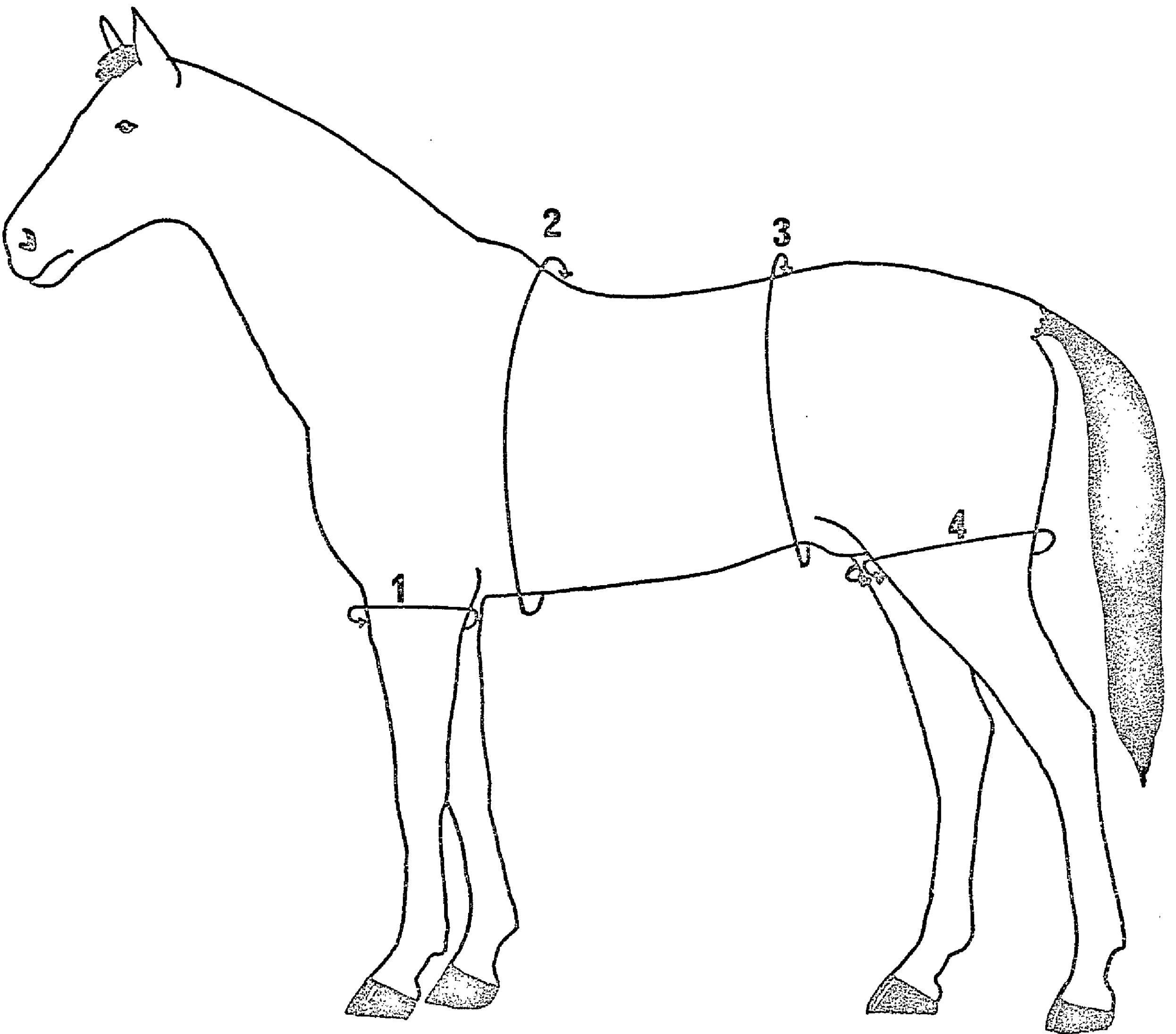
Figure 4.2 Diagrammatic representation of some of the reference points used for the body measurements

1 = upper fore limb

2 = girth

3 = flank

4 = stifle → stifle


1
2
3
4

HISTOCHEMISTRY

Samples for histochemical analysis were taken from the MG and BF and prepared as described in Section 2. Sections were then stained for myosin ATP-ase pH 9.4 and succinate dehydrogenase and classified into fibre types FT, FTH and ST (Section 2). The succinate dehydrogenase sections from the pretraining and post-anaerobic samples were then photographed and used for fibre area analysis (Section 2). MG sections from pretraining, post-aerobic, post anaerobic and 2 and 6 weeks detraining were stained for capillary density and capillaries were counted, according to their oxidative classification (Section 2).

BIOCHEMISTRY

Samples from all 3 muscles at each biopsy time were prepared and analysed as described in Section 2. Water content, LDH, PFK, CS, HAD, Cyt. ox. and βglucuronidase activities, glycogen and total protein concentration were determined. No samples were taken from the Semi-T of horse 4.

RESULTS

In the first part of the cross-over, horse 3 became lame after 3 weeks of anaerobic training and was withdrawn. The lameness was a result of an infection in the fore hoof. In cross-over 2, horse 7 became temporarily lame. The cause of the lameness could not be ascertained and it was withdrawn from the last week of the aerobic work (but was back in use for the anaerobic training). Samples were taken from these horses at the end of their exercise period and as they did not differ from similar samples taken from the other horses at the end of their training period, they were included in the results.

Where no differences existed between the 2 cross-overs, results from both parts of the cross-over were combined to give n = 6 in the control group and n = 6 in the anabolic group.

BODY WEIGHT

The response to training with respect to body weight was different in the 2 cross-overs (Table 4.1). In cross-over 1 all 6 horses decreased their body weight during aerobic training; however the decrease was only significant ($P < 0.01$) in the control group. The body weights continued to decrease with anaerobic training and were significantly lower (control $P < 0.01$, anabolic $P < 0.05$) at the post-anaerobic sample when compared to PT values. Throughout the 15 weeks of detraining, in spite of increasing the feed (an additional 2.7 Kg/day "Complete Cube") the horses did not

TABLE 4.1 The effect of an anabolic steroid on body weight (Kg) of 6 horses throughout a training and detraining period (mean ± S.E.M. n = 3)

BODY WEIGHT

	PT (3)	Anaerobic Training (wk)				D e t r a i n i n g (wk)							
Cross-over 1		1	2	3	5	0	1	2	3	4	5	6	12
Control	518 ± 24	507 ± 21	509 ± 19	504 ± 19	*** 496 ± 24	*** 478 ± 15	*** 472 ± 13	*** 471 ± 13	*** 474 ± 14	*** 475 ± 17	*** 476 ± 16	*** 479 ± 17	*** 490 ± 14
Anabolic	495 ± 25	491 ± 19	494 ± 21	488 ± 21	489 ± 22	* 457 ± 13	* 465 ± 16	* 458 ± 14	* 460 ± 14	* 459 ± 14	* 458 ± 15	(n=2) ** 439 ± 7	* 465 ± 11
Cross-over 2													
Control	472 ± 21	478 ± 22	473 ± 25	477 ± 23	(n = 2) 488 ± 43	462 ± 19	471 ± 21	470 ± 21	470 ± 22	474 ± 22	482 ± 23	478 ± 23	476 ± 18
Anabolic	497 ± 14	494 ± 14	490 ± 19	499 ± 15	495 ± 16	496 ± 17	500 ± 17	493 ± 15	490 ± 14	490 ± 16	489 ± 13	488 ± 15	487 ± 14

* P <0.05 with respect to pretraining values.

*** P <0.01 with respect to pretraining values.

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

regain their pretraining weights. Statistically, changes in the second part of the cross-over are compared to the new pretraining body weights. Although 5 out of the 6 horses decreased their weight over the entire training period of cross-over 2, the decrease was not significant. There was no difference at any stage in cross-over 1 or cross-over 2 between control and anabolic-treated animals.

BODY MEASUREMENTS

Chest and Girth

There was no change in these measurements with respect to training or training/anabolic steroid administration in either of the cross-overs. The results from the cross-overs were therefore combined (Table 4.2) but still did not show any significant changes.

Flank

All the horses in cross-over 1 decreased their flank measurement by the end of the anaerobic training period ($P < 0.01$, $n = 5$) (Table 4.3). Throughout detraining the measurement increased and was not significantly different at pretraining (2) from pretraining (1). No change in cross-over 2 was noted with respect to training. There was no difference in either cross-over between anabolic and control group.

Fore Limb and Stifle to Stifle

These measurements responded similarly in that both increased significantly (fore limb $P < 0.02$, stifle $P < 0.05$) with training in cross-over 1 (Table 4.4). During detraining the fore limb measurements decreased and

TABLE 4.2 The effect of an anabolic steroid on chest and girth measurements (cm) of 6 horses throughout a training and detraining period (mean ± S.E.M. n = 6)

CHEST MEASUREMENTS

	PT (3)	Aerobic Training (wk)		Anaerobic Training (wk)			Detraining (wk)			
		2	4	2	4	6	2	4	6	12
Control	38 ± 1.5	38 ± 1.4	38 ± 1.6	38 ± 1.5	38 ± 1.6	38 ± 1.7	38 ± 1.8	38 ± 1.4	39 ± 1.2	38 ± 1.3
Anabolic	36 ± 1.7	37 ± 1.7	37 ± 1.9	37 ± 1.5	38 ± 1.7	(n = 5) 39 ± 2.3	37 ± 1.7	38 ± 2.0	38 ± 2.1	38 ± 1.8

GIRTH MEASUREMENTS

	PT (3)	Aerobic Training (wk)		Anaerobic Training (wk)			Detraining (wk)			
		2	4	2	4	6	2	4	6	12
Control	184 ± 1.0	182 ± 1.4	181 ± 2.7	182 ± 1.5	182 ± 1.5	182 ± 1.8	185 ± 1.5	186 ± 1.7	184 ± 1.1	184 ± 1.4
Anabolic	185 ± 2.6	183 ± 1.9	183 ± 1.5	184 ± 1.9	182 ± 1.5	182 ± 1.5	185 ± 1.8	185 ± 2.6	187 ± 4.5	185 ± 1.9

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

TABLE 4.3 The effect of an anabolic steroid on flank measurement (cm) of 6 horses during a training and detraining period (mean ± S.E.M.)

FLANK MEASUREMENT

	PT (3)	Aerobic Training (wk)		Anaerobic Training (wk)			Detraining (wk)			
Cross-over 1		2	4	2	4	6	2	4	6	12
Control (n = 3)	176 ± 2.3	181 ± 1.3	178 ± 3.7	171 ± 3.2	167 ± 1.8	164 ± 1.2	170 ± 1.5	172 ± 1.2	172 ± 3.0	175 ± 3.1
Anabolic (n = 3)	181 ± 5.3	181 ± 5.7	181 ± 2.4	173 ± 1.3	170 ± 3.8	(n = 2) 167 ± 2.2	173 ± 2.9	171 ± 5.7	169 ± 4.9	172 ± 4.6
Control + Anabolic (n = 6)	178 ± 2.8	181 ± 2.6	180 ± 2.1	172 ± 1.6	169 ± 2.0	*** 166 ± 1.3	172 ± 1.6	172 ± 2.6	170 ± 2.6	172 ± 2.4
Cross-over 2		2	4	2	4	6	2	4	6	12
Control (n = 3)	174 ± 4.3	176 ± 2.3	177 ± 1.5	169 ± 1.9	177 ± 3.0	173 ± 0.6	180 ± 3.0	183 ± 2.7	186 ± 2.9	182 ± 2.6
Anabolic (n = 3)	180 ± 5.3	175 ± 3.7	177 ± 3.5	173 ± 0.3	175 ± 4.7	175 ± 4.7	178 ± 2.8	185 ± 7.9	184 ± 6.1	182 ± 3.1
Control + Anabolic (n = 6)	178 ± 3.4	176 ± 2.0	177 ± 1.7	171 ± 1.2	176 ± 2.5	174 ± 2.2	179 ± 2.0	184 ± 3.8	185 ± 3.1	182 ± 1.9

*** P <0.01 with respect to pretraining levels (n = 5).

Figures in parenthesis represent the number of occasions the animals were sampled within the period.

TABLE 4.4 The effect of an anabolic steroid on fore limb and stifle to stifle measurement (cm) of 6 horses throughout a training and detraining period (mean ± S.E.M.)

FORE LIMB MEASUREMENT

	PT (3)	Aerobic Training (wk)		Anaerobic Training (wk)			Detraining (wk)			
Cross-over 1		2	4	2	4	6	2	4	6	12
Control (n = 3)	45 ± 0.8	44 ± 0.5	44 ± 0.2	43 ± 0.6	45 ± 0.3	47 ± 1.0	45 ± 1.3	44 ± 0.3	44 ± 0.9	43 ± 0.4
Anabolic (n = 3)	45 ± 1.3	45 ± 1.2	46 ± 1.8	46 ± 1.9	45 ± 0.6	(n = 2) 46 ± 1.2	45 ± 1.5	44 ± 1.2	43 ± 1.5	42 ± 0.9
Control + Anabolic (n = 6)	45 ± 0.7	45 ± 0.6	45 ± 0.9	44 ± 1.0	45 ± 0.3	47 ± 0.7**	45 ± 0.9	44 ± 0.6	44 ± 0.8	43 ± 0.6
Cross-over 2		2	4	2	4	6	2	4	6	12
Control (n = 3)	42 ± 0.7	41 ± 0.9	43 ± 1.2	43 ± 0.9	44 ± 1.5	43 ± 0.6	45 ± 0.0	45 ± 0.0	42 ± 0.5	42 ± 0.4
Anabolic (n = 3)	43 ± 0.7	41 ± 0.9	41 ± 0.6	41 ± 0.7	41 ± 0.7	41 ± 1.0	43 ± 1.0	42 ± 0.9	42 ± 1.2	43 ± 0.5
Control + Anabolic (n = 6)	43 ± 0.4	41 ± 0.6	42 ± 0.7	42 ± 0.8	43 ± 1.0	42 ± 0.7	44 ± 0.6	44 ± 0.7	42 ± 0.6	42 ± 0.4

STIFLE TO STIFLE MEASUREMENT

	PT (3)	Aerobic Training (wk)		Anaerobic Training (wk)			Detraining (wk)			
Cross-over 1		2	4	2	4	6	2	4	6	12
Control (n = 3)	134 ± 1.5	133 ± 12.	132 ± 3.5	133 ± 2.5	(n = 2) 137 ± 1.5	136 ± 2.7	133 ± 2.5	133 ± 1.5	134 ± 1.5	134 ± 1.6
Anabolic (n = 3)	131 ± 2.4	131 ± 2.9	132 ± 1.9	133 ± 2.6	137 ± 1.8	(n = 2) 134 ± 1.0	136 ± 1.9	131 ± 3.2	130 ± 1.5	130 ± 2.6
Control + Anabolic (n = 6)	132 ± 1.5	132 ± 1.5	132 ± 1.8	133 ± 1.6	137 ± 1.1*	135 ± 1.6*	135 ± 1.6*	132 ± 1.6	132 ± 1.2	132 ± 1.9
Cross-over 2		2	4	2	4	6	2	4	6	12
Control (n = 3)	130 ± 0.9	125 ± 0.6	123 ± 1.8	130 ± 4.0	129 ± 2.2	131 ± 2.3	129 ± 0.6	126 ± 2.1	131 ± 3.1	130 ± 2.9
Anabolic (n = 3)	135 ± 3.2	127 ± 0.9	127 ± 3.5	128 ± 2.0	130 ± 1.2	129 ± 2.3	127 ± 4.7	125 ± 2.9	129 ± 2.7	132 ± 1.6
Control + Anabolic (n = 6)	133 ± 1.8	126 ± 0.6	125 ± 1.9	129 ± 2.1	130 ± 1.1	130 ± 1.5	128 ± 2.2	126 ± 1.6	130 ± 1.9	131 ± 1.9

* P <0.05 with respect to pretraining value (n = 6)
** P <0.02 with respect to pretraining value (n = 5)

Figures in paranthesis represent the number of occasions the animals were sampled within the period.

pretraining (2) values were significantly different ($P < 0.01$) from pretraining (1). No change was noted in cross-over 2. There was no difference in either cross-over between anabolic and control group.

URINE ANALYSES (Figure 4.3)

Volume

Urine volume increased significantly in both cross-overs during the training period. There was no difference between control and anabolic group.

Total creatinine

Twenty-four hour urinary total creatinine did not alter significantly throughout the period of investigation nor was there any difference between the control and anabolic group.

Nitrogen

An increase in nitrogen excretion was noted in all animals in both parts of the cross-over during the training period. However, the cross-overs differed in that in cross-over 1 the mean nitrogen excretion during the total training period was significantly less ($P < 0.02$) in the anabolic group 121.3 ± 7.7 g/24 hrs., than in the control, 150.9 ± 8.2 g/24 hrs. No such effect was noted in cross-over 2.

MUSCLE

Histochemistry

A similar result was found in both parts of the cross-over for all the histochemical parameters measured, and the results have therefore been combined. The MG

Figure 4.3 The effect of an anabolic steroid on several urinary parameters throughout a training and detraining period (n = 6 except the nitrogen results where n = 3)

Training Detraining

1 2

Urine Volume

Control
Anabolic

L/24Hrs

20
10

Total Urinary Creatinine

mM/24hrs.

200
100
0

Total Urinary Nitrogen

g/24 hrs.

200
100

CROSS OVER 1

g/24 hrs.

200
100
0

CROSS OVER 2

-2 0 5 11
WEEKS 0 6 12

showed a similar pattern in the control and anabolic group (Figure 4.4). The percentage FTH fibres increased progressively with training and were significantly different ($P < 0.02$) from pretraining values at the end of anaerobic training.

The FT fibres showed a reciprocal decline ($P < 0.05$ control, $P < 0.05$ anabolic) at the post-anaerobic sample.

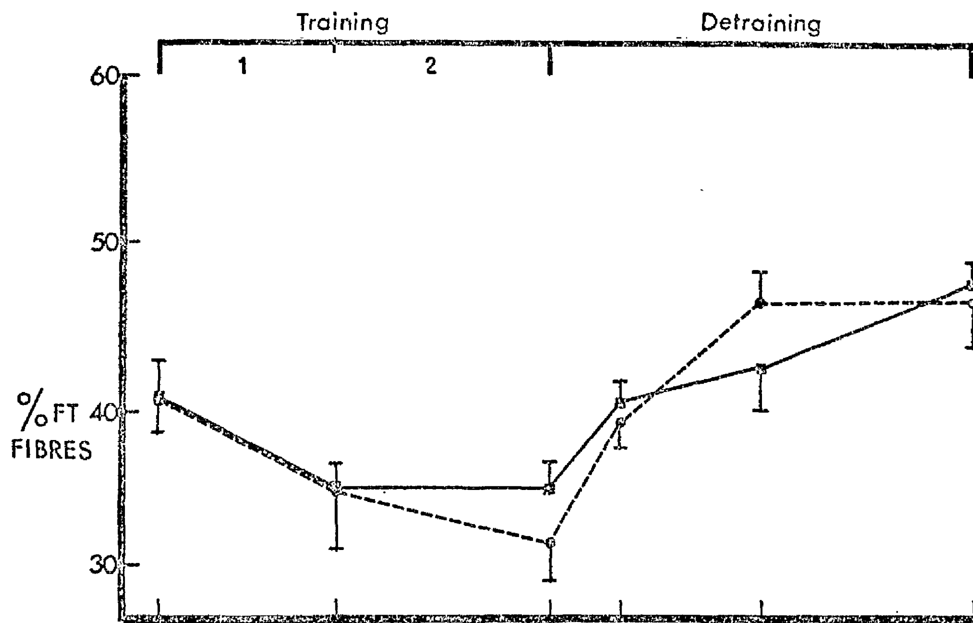
There was no change in the percentage ST fibres with respect to training or training/anabolic steroid administration.

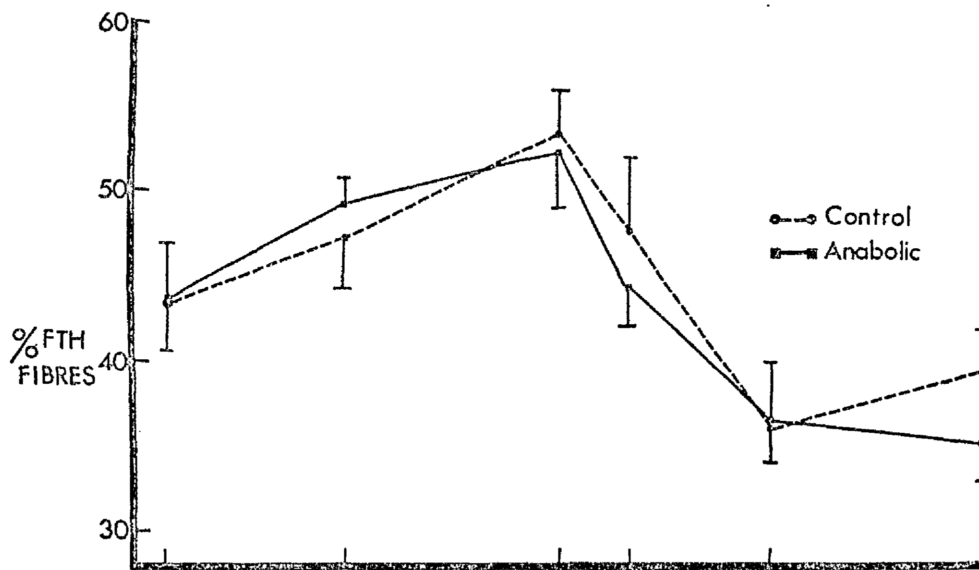
The percentage FTH fibres in the control group of the BF increased after aerobic training ($P < 0.05$) (Figure 4.5) and was still significantly greater ($P < 0.05$) at the post-anaerobic stage. In the anabolic group although the percentage FTH fibres increased significantly with aerobic training ($P < 0.05$) the post-anaerobic training sample was not significantly different from pretraining values. The sample taken at this time also showed a significant difference between the anabolic and control groups ($P < 0.05$).

The FT fibres of the control group decreased significantly ($P < 0.05$) at the post-anaerobic sample. The FT fibres of the anabolic group did not alter significantly. At the post-anaerobic sample there was a significant difference between control and anabolic group ($P < 0.05$).

There was no change in the percentage ST fibres with respect to training or training/anabolic steroid

Figure 4.4 The effect of an anabolic steroid on the percentage fibre composition of the MG throughout a training and detraining period (n = 6)


Training
Detraining
1
2
60
50
40
30
%FT
FIBRES


60
50
40
30
%FTH
FIBRES
Control
Anabolic

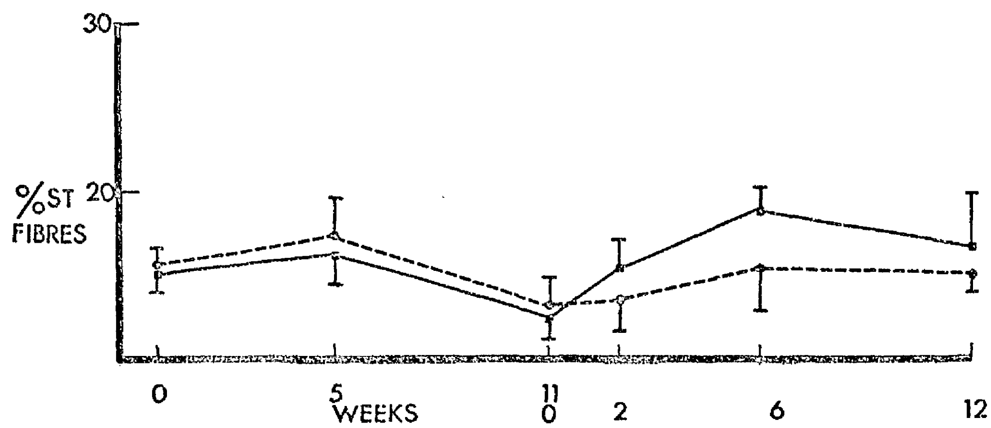
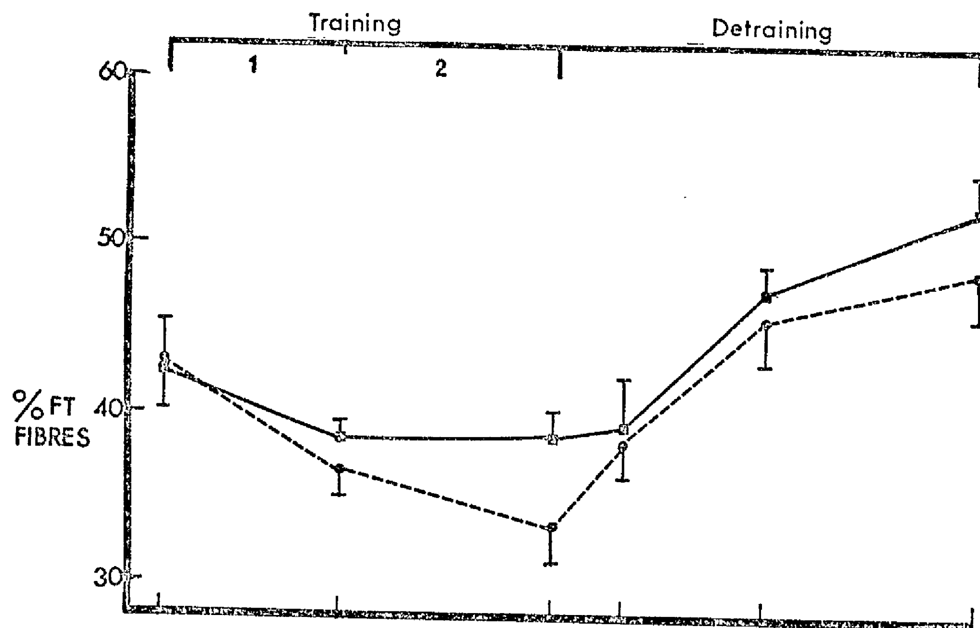
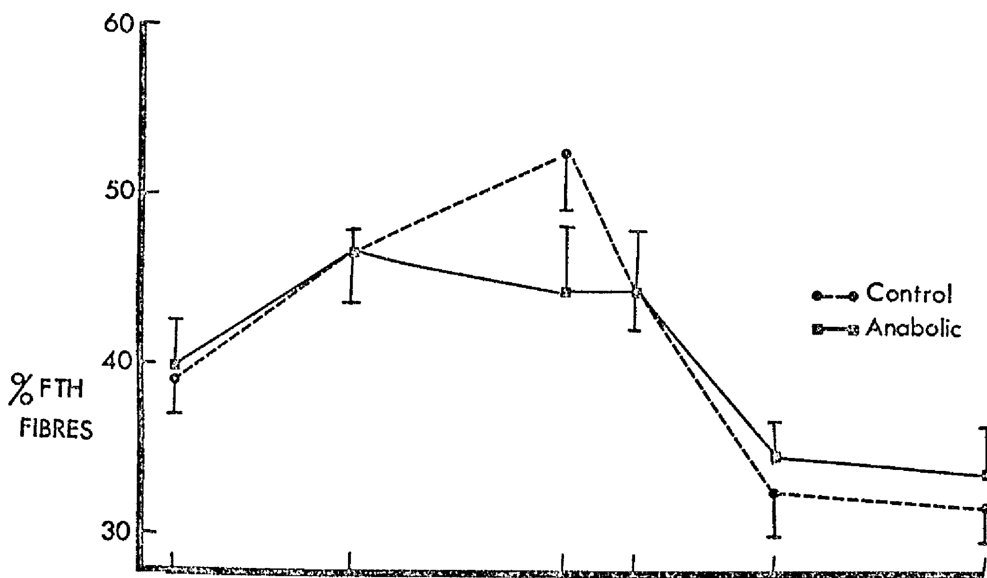
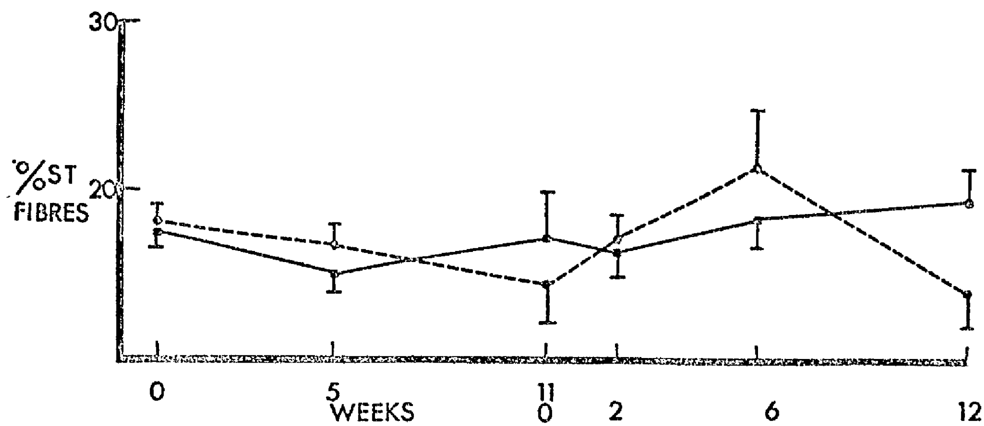

30
20
%ST
FIBRES
0
5
WEEKS
11
0
2
6
12

Figure 4.5 The effect of an anabolic steroid on the percentage fibre composition of the BF throughout a training and detraining period (n = 6)


Training
Detraining
1
2
60
50
40
30
%FT
FIBRES


60
50
40
30
%FTH
FIBRES
Control
Anabolic


30
20
%ST
FIBRES
0
5
WEEKS
11
0
2
6
12

administration.

FIBRE AREAS

In both the MG and BF the LO fibres were significantly larger ($P < 0.01$) than the HO fibres in both control and anabolic group (Figure 4.6). Fibre areas were similar in both muscles and were unaffected by training or training/anabolic administration (Figure 4.7).

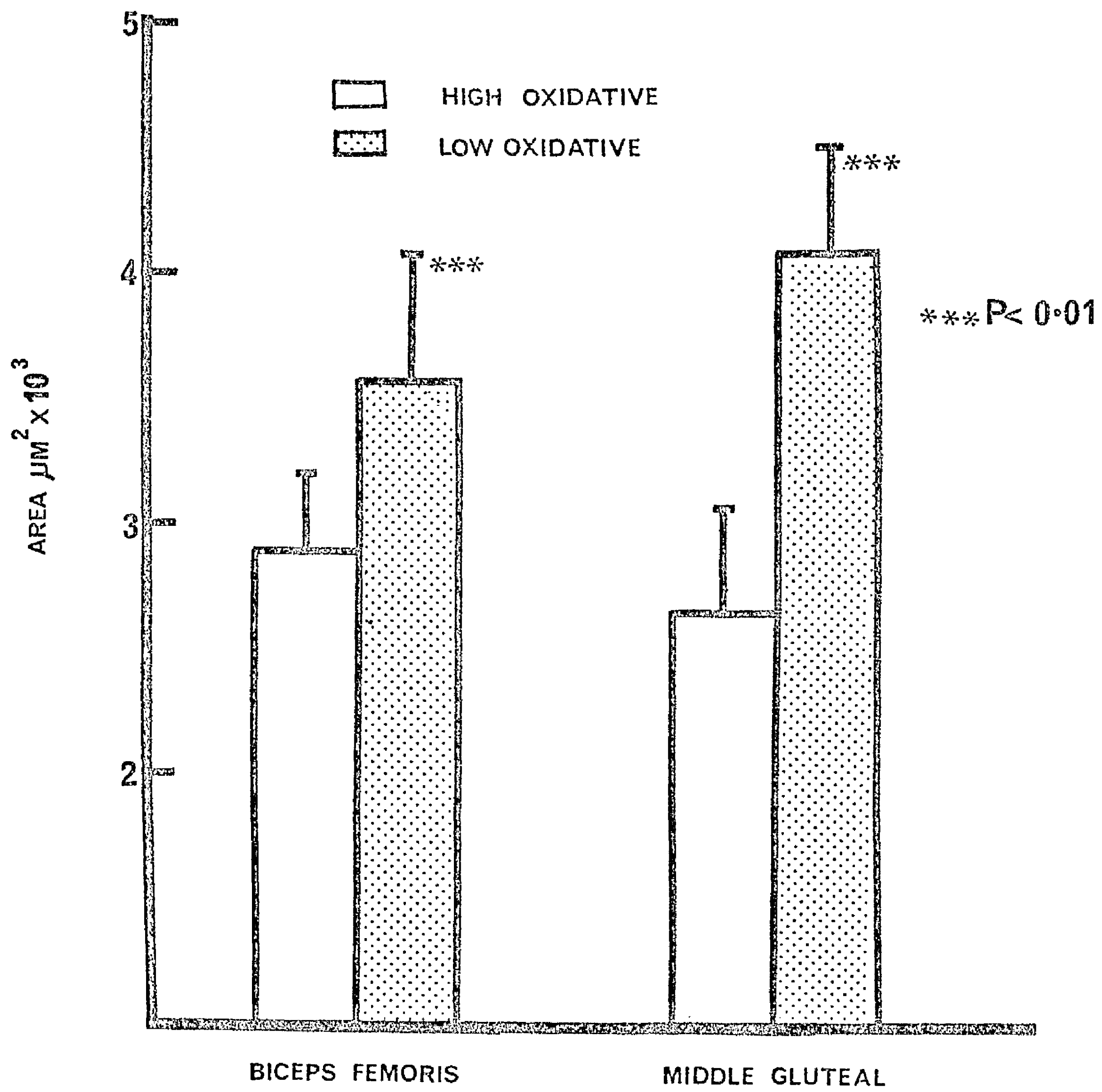
To reduce the error, introduced by the possible contraction of the fibres at the time of biopsy, the areas are also expressed as a ratic of HO:LO (Table 4.5). The MG did not show any alterations with training but the BF increased its fibre ratio significantly ($P < 0.05$).

The mean area measurements can be combined with the mean histochemical fibre typing to give the relative area occupied by each fibre type within the muscle (Table 4.6). The percentage area occupied by the HO fibres increased in both muscles in control and anabolic groups with training ($P < 0.05$).

CAPILLARY DENSITY

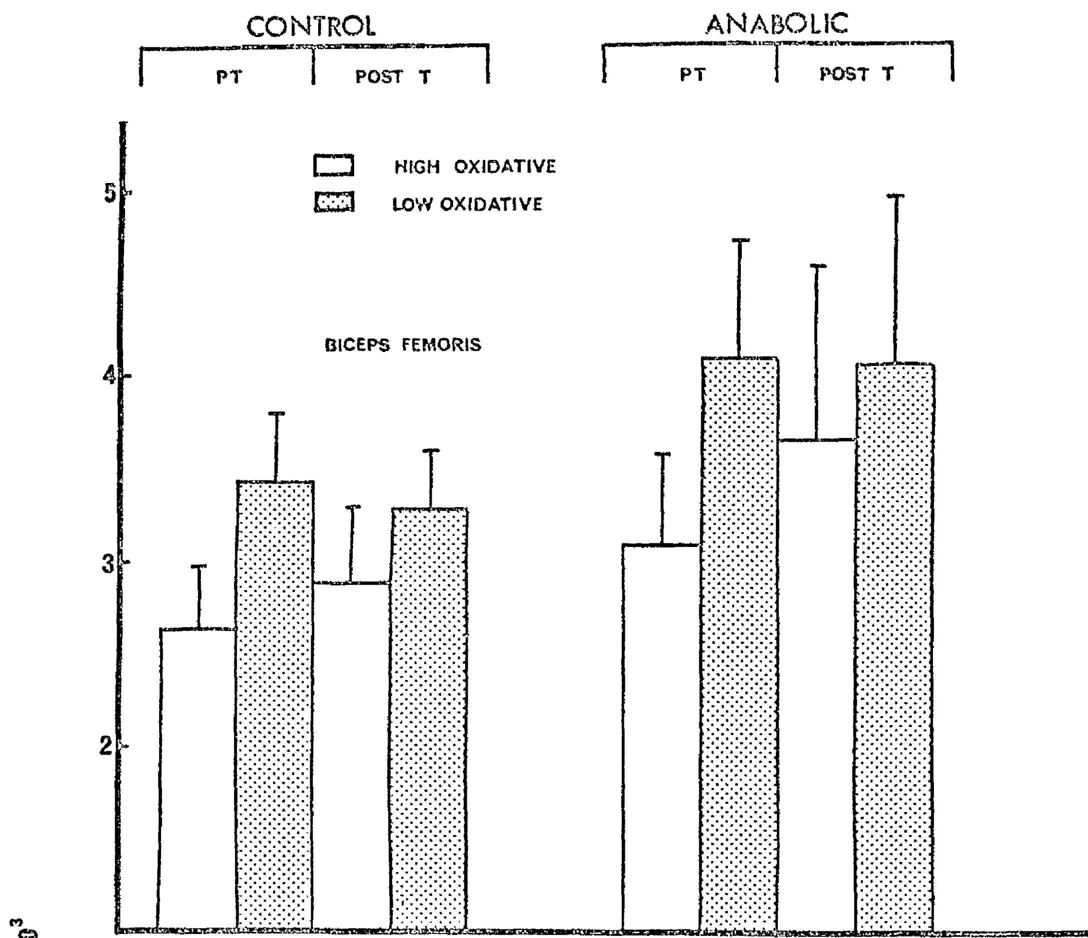
Before training the number of capillaries around each fibre type, LO and HO, did not differ significantly. With aerobic training the number of capillaries around the HO fibres increased ($P < 0.05$) in both control and anabolic steroid group but was not significantly different from pretraining values at the post-anaerobic sample (Table 4.7). The number of capillaries around the LO fibres increased

Figure 4.6 Areas of LO and HO fibres of the MG and BF at rest (n = 6)



5
4
3
2
AREA $\mu M^2 \times 10^3$
HIGH OXIDATIVE
LOW OXIDATIVE
***
***
*** P< 0·01
BICEPS FEMORIS
MIDDLE GLUTEAL

Figure 4.7 The effect of 11 weeks training and 11 weeks training/anabolic steroid administration on the fibre areas of HO and LO fibres of the MG and BF (n = 6)


CONTROL
PT
POST T
ANABOLIC
PT
POST T
HIGH OXIDATIVE
LOW OXIDATIVE
BICEPS FEMORIS
5
4
3
2
AREA µm² x 10³

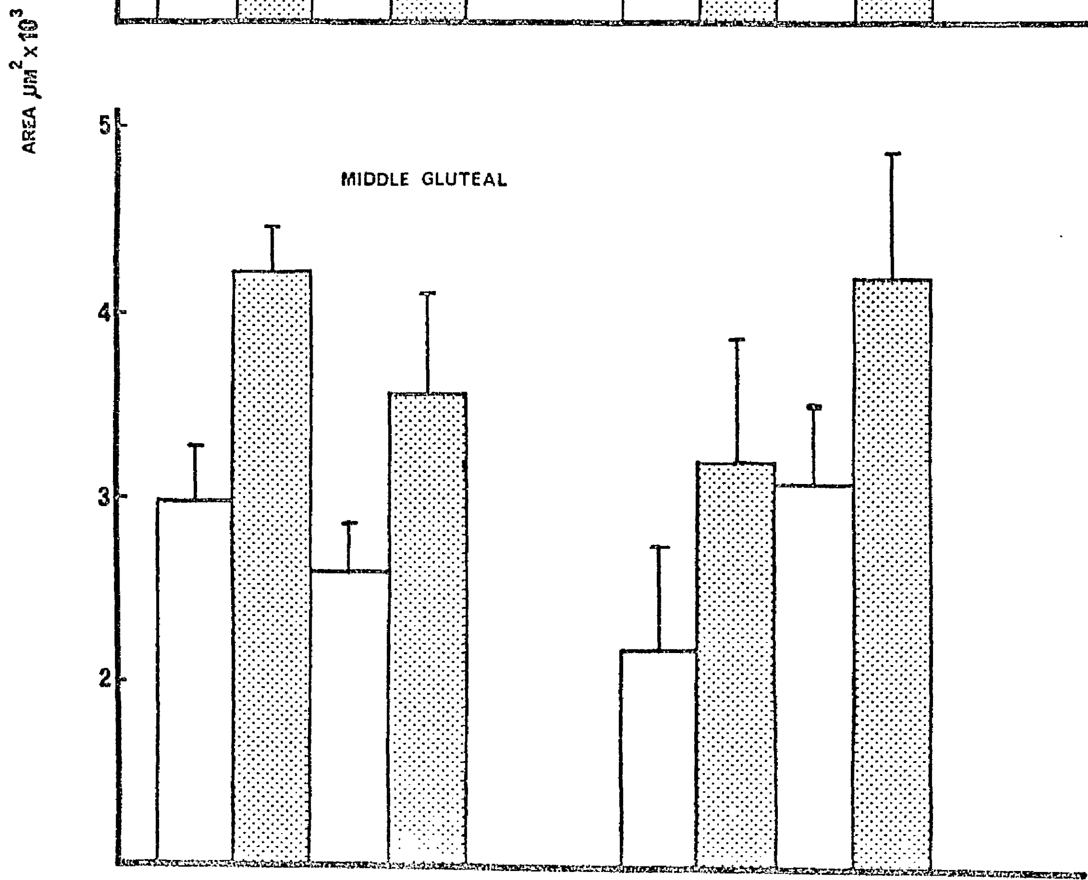

MIDDLE GLUTEAL
5
4
3
2

TABLE 4.5 The effect of an anabolic steroid on fibre areas, expressed as a ratio H.O./L.O. before and after an 11 week training period

Middle Gluteal

	Control		Anabolic	
Horse	PT	Post-anaerobic	PT	Post-anaerobic
1	0.42	0.57	0.59	0.62
2	0.72	0.75	0.64	0.94
3	0.85	0.81	0.75	0.89
4	0.81	0.80	0.47	0.69
5	0.80	0.80	0.75	0.60
6	0.76	0.87	0.64	0.72
$\bar{x}$±S.E.M.	0.72±0.05	0.76±0.02	0.64±0.06	0.74±0.06

Biceps Femoris

	Control		Anabolic	
Horse	PT	Post-anaerobic	PT	Post-anaerobic
1	0.78	0.73	0.83	0.87
2	0.68	0.87	0.90	1.30
3	0.81	0.98	0.76	0.99
4	0.74	1.31	0.69	0.77
5	0.80	0.85	0.76	1.07
6	0.82	0.81	0.63	0.85
$\bar{x}$±S.E.M.	0.77±0.02	0.93±0.09	0.76±0.04	0.97*±0.08

* P <0.05 compared to pretraining value

TABLE 4.6 The effect of an anabolic steroid on relative percentage areas of fibre types within a muscle (mean ± S.E.M. n = 6)

Muscle	Fibre Type	Control PT	Control Post-anaerobic	Anabolic PT	Anabolic Post-anaerobic
MG	H.O.	49.9 ± 4.1	58.2 ± 2.5*	47.0 ± 2.2	59.0 ± 1.6*
	L.O.	52.0 ± 4.1	41.8 ± 2.5*	52.9 ± 2.2	40.9 ± 1.6*
BF	H.O.	50.6 ± 2.0	63.1 ± 3.9*	50.3 ± 2.4	57.8 ± 1.2*
	L.O.	49.4 ± 2.0	36.9 ± 3.9*	49.7 ± 2.4	42.1 ± 1.2*

P <0.05 when compared to pretraining value.

TABLE 4.7 The effect of an anabolic steroid on the capillary number around each fibre type HO or LO of the MG throughout a training period (Mean ± S.E.M. n = 6)

	Fibre Type	PT	Post-aerobic	Post-anaerobic	Detraining 2	Detraining 6
Control	H.O.	4.4 ± 0.3	5.3 ± 0.3*	4.7 ± 0.4	4.5 ± 0.2	3.9 ± 0.3
	L.O.	4.5 ± 0.3	5.6 ± 0.3	5.2 ± 0.4	4.6 ± 0.2	4.2 ± 0.3
Anabolic	H.O.	4.2 ± 0.3	5.6 ± 0.5*	5.5 ± 0.5	4.5 ± 0.4	4.0 ± 0.4
	L.O.	4.3 ± 0.2	5.4 ± 0.5*	5.6 ± 0.5*	4.4 ± 0.3	4.5 ± 0.4

* P <0.05 with respect to PT value.

TABLE 4.8 The effect of an anabolic steroid on the number of capillaries around a fibre type of the MG relative to its fibre area ($\mu m^2 \times 10^3$) throughout a training period (Mean ± S.E.M. n = 6)

Fibre Type	Control PT	Control Post-aerobic	Control Post-Anaerobic	Anabolic PT	Anabolic Post-aerobic	Anabolic Post-anaerobic
H.O.	1.6 ± 0.2*	1.4 ± 0.3	1.9 ± 0.3	2.3 ± 0.5*	2.0 ± 0.7	1.9 ± 0.3
L.O.	1.2 ± 0.08	1.3 ± 0.2	1.6 ± 0.3	1.4 ± 0.2	1.7 ± 0.4	1.4 ± 0.9

P <0.05 H.O. versus L.O.

significantly ($P < 0.05$) in the anabolic steroid treated animals at both the post-aerobic and post-anaerobic stage. There was no significant change in the controls although 5 out of the 6 animals had increased at the post-aerobic sample.

Capillary number increased with the size of the fibre in both HO fibres ($r = 0.53$, $n = 150$) and LO fibres ($r = 0.48$, $n = 150$) in the animal at rest. The number of capillaries can therefore be expressed by relating the number to the fibre area. A significant difference ($P < 0.05$) between the capillary number around each fibre type at rest in both the anabolic and control group was now evident (Table 4.8). However, in response to training, the capillary number/ fibre area did not change in the anabolic or control group at any of the sample points during training (Table 4.8).

BIOCHEMISTRY

The response to training and anabolic steroids was similar in both parts of the cross-over and the results from cross-over 1 and cross-over 2 have therefore been combined.

WATER CONTENT

The water content of the 3 muscles did not alter with respect to training/anabolic steroid treatment or to training (Table 4.9).

TOTAL PROTEIN

The total protein content of the 3 muscles studied did not alter with respect to training or to training/ anabolic steroid administration (Table 4.9).

TABLE 4.9 The effect of an anabolic steroid on percentage water content and total protein content of 3 skeletal muscles of 6 Thoroughbred horses throughout a training and detraining period (mean ± S.E.M. n = 17)

		PT	Post-aerobic	Post-anaerobic	Detraining 2	Detraining 4	Detraining 6	Detraining 9	Detraining 12
% H_2O Content	Control	74.0 ± 0.7	74.2 ± 0.6	73.4 ± 0.4	74.2 ± 0.4	74.4 ± 0.4	74.4 ± 0.3	73.7 ± 0.4	73.2 ± 0.6
	Anabolic	73.6 ± 0.5	73.7 ± 0.5	73.6 ± 0.4	73.9 ± 0.5	74.4 ± 0.7	74.1 ± 0.5	74.0 ± 0.6	74.2 ± 0.8
Total Protein mg/g dry wt tissue	Control	582 ± 17.9	593 ± 21.8	551 ± 26.0	570 ± 25.2	565 ± 25.9	581 ± 21.0	577 ± 22.9	560 ± 21.4
	Anabolic	545 ± 17.6	562 ± 21.0	524 ± 21.7	566 ± 19.4	545 ± 19.6	536 ± 22.5	535 ± 20.6	569 ± 22.3

GLYCOGEN CONTENT

There was no significant change in glycogen concentration of any of the muscles at the post-aerobic sample. All control muscles showed an increased concentration and were significantly different from pretraining levels ($P < 0.01$) at the anaerobic stage. Of the anabolic group only the semi-T and the MG increased significantly ($P < 0.05$). The BF was not significantly altered throughout the training period although 5 out of the 6 animals had in fact increased.

There was no difference at any sample point between control and anabolic group (Figure 4.8).

ENZYME ACTIVITIES

Because of the small numbers and the large variation found in the enzyme determinations from skeletal muscle undergoing training, the 3 muscles have been combined for each enzyme to give a mean value (Tables 4.10-4.15). The mean values of the 3 muscles are presented in the written text of the 'Results' with reference only to any pertinent point in individual muscles.

LDH AND PFK (Figure 4.9)

Training

Neither LDH nor PFK was altered by training or by anabolic steroid administration. The anabolic group of the semi-T decreased in PFK activity significantly ($P < 0.05$).

Detraining

The control group did not show altered activity. However the LDH and PFK activity of the anabolic steroid

Figure 4.8 The effect of an anabolic steroid on the glycogen concentration of the MG, semi-T and BF throughout a training and detraining period (n = 6)

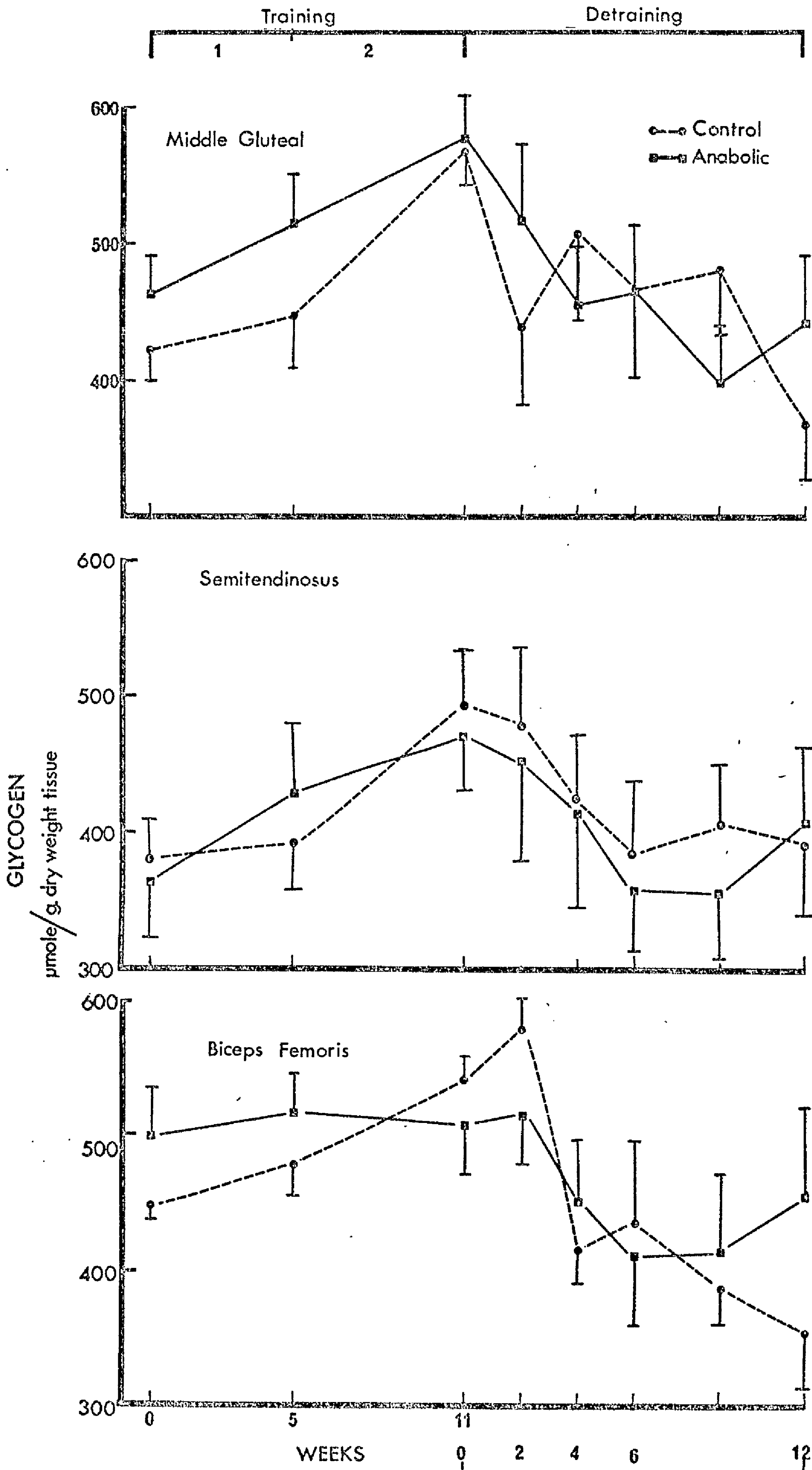

Training
Detraining
1
2
Middle Gluteal
Control
Anabolic
600
500
400
Semitendinosus
600
500
400
300
GLYCOGEN
µmole/g dry weight tissue
Biceps Femoris
600
500
400
300
0
5
11
WEEKS
0
2
4
6
12

TABLE 4.10 The effect of an anabolic steroid on the activity of LDH (μmoles x 10^2/min/g) throughout a training and detraining period (Mean ± S.E.M.)

Muscle		PT	Post-aerobic	Post-anaerobic	Detraining 2	Detraining 4	Detraining 6	Detraining 9	Detraining 12
MG n = 6	Control	18.0 ± 1.9	20.0 ± 2.6	16.6 ± 1.5	16.6 ± 1.5	17.7 ± 1.8	16.0 ± 3.1	17.1 ± 2.2	14.2 ± 2.6
	Anabolic	18.3 ± 2.1	18.5 ± 2.3	22.7 ± 4.2	16.2 ± 1.6	19.4 ± 3.1	17.0 ± 1.5	15.8 ± 2.4	11.8** ± 1.4
BF n = 6	Control	19.3 ± 1.9	21.1 ± 1.6	16.7 ± 1.7	22.4 ± 2.5	19.3 ± 1.5	18.0 ± 1.6	14.9 ± 1.8	15.5 ± 2.9
	Anabolic	18.2 ± 1.2	22.5 ± 3.1	19.5 ± 1.5	18.0 ± 1.6	20.3 ± 2.5	20.5 ± 1.8	18.7 ± 2.7	15.2* ± 1.0
Semi-T n = 5	Control	25.6 ± 2.9	22.1 ± 3.5	23.6 ± 4.3	22.0 ± 3.7	27.2 ± 3.3	28.1 ± 2.5	30.1 ± 5.7	30.1 ± 3.9
	Anabolic	28.1 ± 5.2	29.0 ± 6.1	22.3 ± 2.5	23.6 ± 4.1	30.7 ± 4.7	23.6 ± 2.6	22.8 ± 1.9	23.3 ± 5.6
$\bar{x}$ n = 17	Control	20.7 ± 1.4	21.0 ± 1.4	18.7 ± 1.6	20.0 ± 1.6	21.0 ± 1.6	20.3 ± 1.8	20.1 ± 2.4	19.4 ± 2.4
	Anabolic	21.2 ± 2.0	23.0 ± 2.4	21.5 ± 1.7	19.0 ± 1.5	23.1 ± 2.2	20.2 ± 1.2	18.8 ± 1.5	16.4 ± 2.0*

* $P < 0.05$ compared to pretraining level

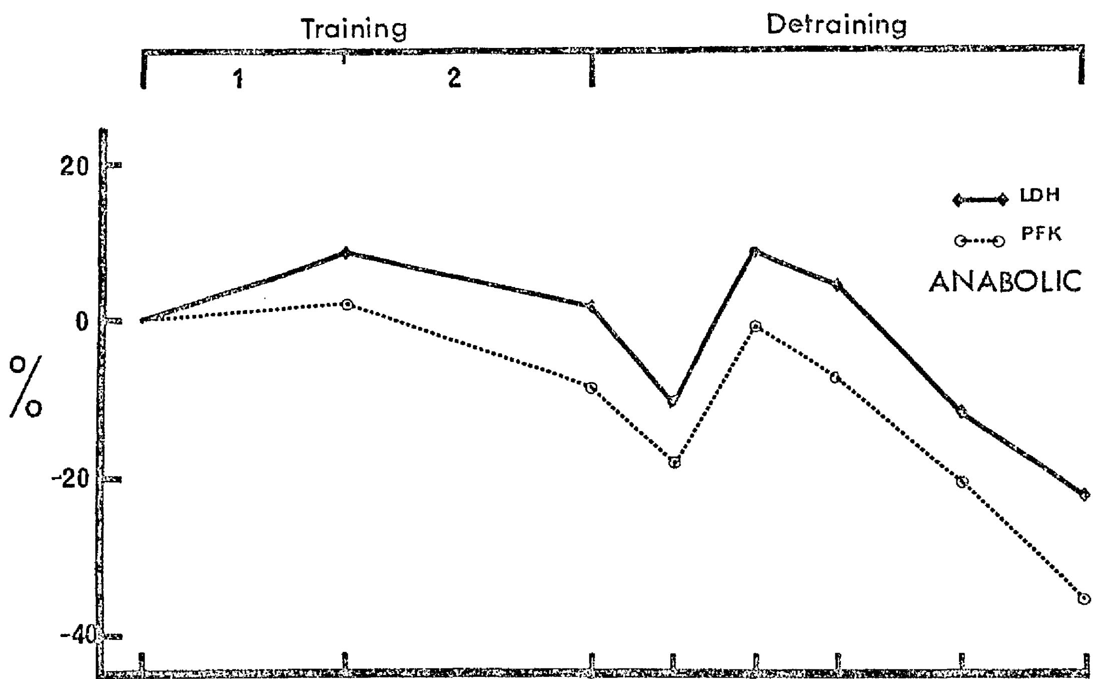
** $P < 0.02$ compared to pretraining level.

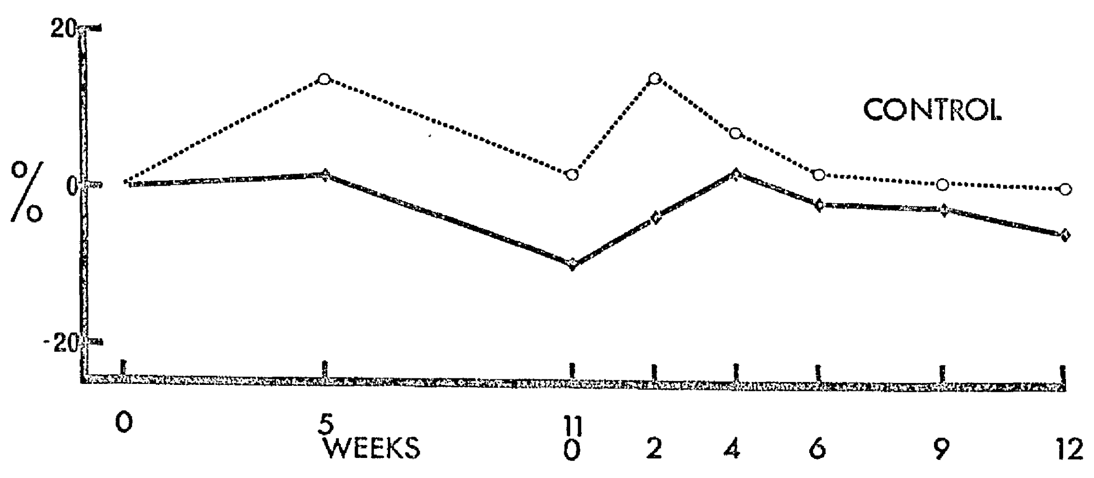
TABLE 4.11 The effect of an anabolic steroid on the activity of PFK (μmoles min/g) throughout a training and detraining period (Mean ± S.E.M.)

Muscle		PT	Post-aerobic	Post anaerobic	Detraining 2	4	6	9	12
MG n = 6	Control	40.7 ± 4.5	43.6 ± 4.5	37.6 ± 6.3	43.7 ± 6.1	42.8 ± 4.3	40.5 ± 9.1	37.2 ± 3.8	34.2 ± 6.6
	Anabolic	43.1 ± 2.8	38.9 ± 6.4	49.4 ± 6.3	37.4 ± 7.7	42.7 ± 5.6	38.5 ± 7.0	36.1 ± 8.1	25.9 ± 3.3*
BF n = 6	Control	33.8 ± 5.1	41.0 ± 3.4	34.2 ± 3.4	49.4 ± 2.8	34.8 ± 4.2	32.3 ± 5.5	28.1 ± 4.1	31.9 ± 6.0
	Anabolic	34.1 ± 4.0	40.2 ± 7.4	32.4 ± 4.1	30.6 ± 4.9	36.8 ± 4.1	42.0 ± 5.1	32.1 ± 5.4	23.3 ± 1.3*
Semi-T n = 5	Control	39.7 ± 8.2	44.9 ± 9.3	44.6 ± 6.5	35.5 ± 6.3	44.4 ± 5.9	44.1 ± 5.2	50.9 ± 7.0	49.3 ± 6.8
	Anabolic	51.4 ± 9.4	47.1 ± 6.9	33.9 ± 6.4*	36.3 ± 7.7	48.6 ± 7.5	36.7 ± 4.9	33.5 ± 6.9	34.6 ± 8.8
$\bar{X}$ n = 17	Control	38.0 ± 3.3	43.2 ± 3.2	38.8 ± 3.1	42.9 ± 3.1	40.5 ± 2.8	39.0 ± 4.0	38.7 ± 3.5	38.5 ± 3.9
	Anabolic	42.9 ± 3.5	42.1 ± 3.9	38.6± 3.7	34.8± 3.7	42.7 ± 3.3	39.2 ± 3.2	34.0 ± 3.8	27.9 ± 2.9*

* P <0.05 when compared to pretraining values.

Figure 4.9 The effect of an anabolic steroid on levels of LDH and PFK as a percentage of pre-training levels. (n = 17) throughout a training and detraining period (n = 6)


Training
Detraining
1
2
20
0
%
-20
-40
LDH
PFK
ANABOLIC


20
%
0
-20
CONTROL
0
5
WEEKS
11
0
2
4
6
9
12

group increased significantly between the 2 and 4 week detraining samples. The enzymes' activity then decreased with the 12 week detraining sample being significantly lower ($P < 0.05$) than the pretraining value. However pretraining (2) was not significantly different from pretraining (1). This pattern was noted in all the muscles.

The correlation coefficient between LDH and PFK at rest was $r = 0.69$, $n = 17$. The activity ratio did not alter significantly throughout the training.

CS (Figure 4.10)

Training induced an increase in CS activity ($P < 0.05$) at the time of the post-anaerobic sample in the anabolic steroid group. It then gradually decreased throughout detraining. The pattern was similar in all 3 muscles.

Although the MG had shown a significant increase at the time of the post-aerobic sample (Table 4.12) overall the control group did not alter throughout the period of investigation.

HAD (Figure 4.10)

The semi-T had a significantly lower ($P < 0.02$) HAD activity than the other 2 muscles.

Training induced a significant increase ($P < 0.05$) at the post-anaerobic stage in the anabolic steroid group. It then decreased gradually until it was significantly lower than pretraining levels at 12 weeks detraining. The pattern was similar in all 3 muscles.

Although the control group of the MG had shown a

TABLE 4.12 The effect of an anabolic steroid on the activity of CS (µmoles min/g) throughout a training and detraining period (Mean ± S.E.M.)

Muscle		PT	Post-aerobic	Post-anaerobic	Detraining 2	4	6	9	12
MG	Control	6.7 ± 0.5	10.4 ± 1.3*	10.4 ± 1.6	8.6 ± 1.0	7.8 ± 0.76	8.6 ± 1.1	6.9 ± 1.7	6.8 ± 1.7
n = 6	Anabolic	8.6 ± 1.5	11.0 ± 1.7	11.1 ± 1.4	7.6 ± 0.7	6.9 ± 0.6	8.0 ± 1.3	7.2 ± 1.4	7.1 ± 1.6
BF	Control	12.6 ± 1.8	12.1 ± 1.3	12.6 ± 1.5	17.4 ± 3.1	10.3 ± 2.3	11.8 ± 2.7	9.1 ± 1.9	9.1 ± 2.5
n = 6	Anabolic	11.6 ± 0.7	12.7 ± 1.4	13.1 ± 1.5	13.0 ± 0.7	12.0 ± 2.1	11.9 ± 1.6	9.9 ± 1.3	9.3 ± 2.3
Semi-T	Control	8.9 ± 1.6	7.3 ± 0.7	9.4 ± 2.3	8.4 ± 1.1	10.3 ± 1.3	8.7 ± 1.3	9.8 ± 1.5	9.9 ± 1.1
n = 5	Anabolic	7.3 ± 1.4	8.9 ± 1.1	9.3 ± 1.3	7.0 ± 1.0	8.5 ± 1.2	7.7 ± 1.4	7.8 ± 1.2	6.5 ± 0.7
$\bar{x}$	Control	9.4 ± 1.0	9.9 ± 0.8	10.9 ± 1.0	11.7 ± 1.6	9.5 ± 0.9	9.8 ± 1.1	8.5 ± 1.0	8.5 ± 1.1
n = 17	Anabolic	9.3 ± 0.8	11.0 ± 0.9	11.3 ± 0.9*	9.3 ± 0.8	9.2 ± 1.0	9.3 ± 0.9	8.4 ± 0.8	7.7 ± 1.0

* $P < 0.05$ when compared to pretraining value.

TABLE 4.13 The effect of an anabolic steroid on the activity of HAD (μmoles min/g) throughout a training and detraining period (Mean ± S.E.M.)

Muscle		PT	Post-aerobic	Post-anaerobic	Detraining 2	4	6	9	12
MG n = 6	Control	220.7 ± 26.7	285.7 ± 3.3	277 ± 21.1	239.5 ± 12.7	241.7 ± 30.0	232.3 ± 35.2	211.1 ± 24.9	209.2 ± 3.
	Anabolic	240.8 ± 31.7	268.5 ±31.3	285.7 ± 21.6	231.2 ± 30.5	228.7 ± 26.0	239.3 ± 43.6	223.3 ± 45.3	219.3 ± 24.
BF n = 6	Control	159.7 ± 16.7	160.7 ± 20.4	163.7 ± 15.4	184.0 ± 28.0	160.0 ± 27.0	167.8 ± 26.9	144.7 ± 17.3	137.0 ± 26.
	Anabolic	157.5 ± 13.5	171.1± 17.0	187.3 ± 17.2	161.0 ± 16.0	177.5 ± 22.9	151.1 ± 14.6	121.1*** ± 13	107.5*** ± 1.
Semi-T n = 5	Control	68.0 ± 13.0	53.0 ± 3.6	74.8 ± 16.9	68.8 ± 8.6	78.6 ± 10.0	62.8 ± 9.7	76.4 ± 10.7	78.6 ± 9.
	Anabolic	68.0 ± 14.7	76.8 ±10.1	74.2 ± 13.8	58.4 ± 12.1	63.4 ± 10.2	50.8 ± 8.7	66.4 ± 10.7	52.8 ± 7.
$\bar{X}$ n = 17	Control	154.2 ± 18.7	173.1 ±26.7	177.4 ± 22.6	169.7 ± 20.2	164.9 ± 21.3	159.7 ± 22.6	148.0 ± 17.1	145.3 ± 19.
	Anabolic	160.6 ± 21.1	177.8 ± 22.7	188.7 ± 23.4*	155.7 ± 21.0	162.0 ± 20.5	152.8 ± 24.4	141.1 ± 22.7	123.8**** ± 18.

* P <0.05 when compared to pretraining levels
** P <0.02 when compared to pretraining levels
*** P <0.01 when compared to pretraining levels
**** P <0.001 when compared to pretraining levels.

significant increase at the time of the post-aerobic sample (Table 4.13) overall, the control group did not alter throughout the period of investigation.

CYTOCHROME OXIDASE (Figure 4.10)

Both anabolic and control group increased the Cyt. ox. activity with aerobic training (control $P < 0.05$, anabolic $P < 0.02$). This was maintained but not increased with anaerobic training. The pattern was similar in all muscles. There was no significant change in the detraining period.

β-*GLUCURONIDASE* (Figure 4.11)

Training

Training did not affect this enzyme's activity in either the control or anabolic group.

Detraining

The activity in the 2 week detraining sample of the MG was significantly higher ($P < 0.02$) than the pretraining value, although overall the activity of this sample was not significantly higher. The 2 week detraining sample was however significantly higher ($P < 0.01$, $n = 17$) than the 4 week sample. The pattern was followed in the 3 muscles.

Although a similar trend was noted in the control group no significant changes were noted.

The results of Section 4 are summarized in Tables 4.16 and 4.17.

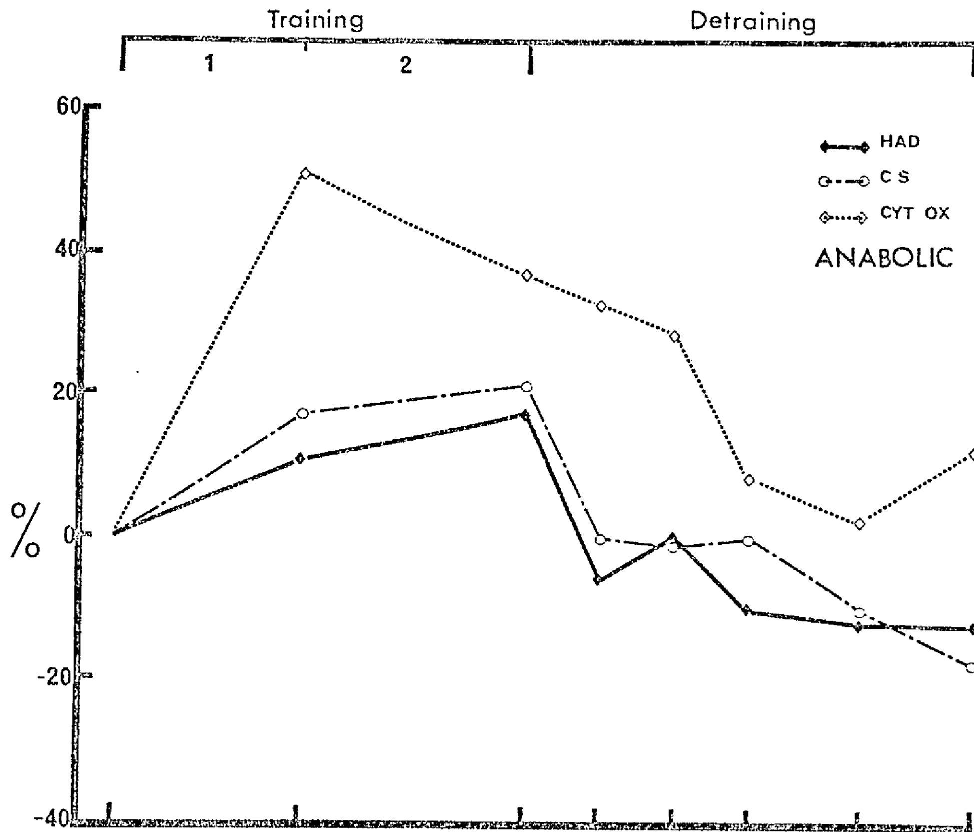
TABLE 4.14 The effect of an anabolic steroid on the activity of Cyt. Ox. (Δ log ferrocytochrome C /min. for a 1:10,000 dry wt. tissue dilution) throughout a training and detraining period (Mean ± S.E.M.)

Muscle		PT	Post-aerobic	Post-anaerobic	Detraining 2	4	6	9	12
MG n = 6	Control	8.1 ± 1.6	12.2 ± 1.6*	13.9 ± 2.1	9.7 ± 1.1	10.3 ± 3.0	6.9 ± 1.7	8.3 ± 1.1	7.2 ± 1.6
	Anabolic	7.4 ± 1.1	14.7 ± 3.2	15.8 ± 3.4	9.3 ± 3.3	9.5 ± 2.1	9.6 ± 2.5	8.6 ± 2.5	6.2 ± 1.1
BF n = 6	Control	10.0 ± 1.6	13.9 ± 2.3	14.8 ± 2.5	14.8 ± 2.0	8.9 ± 1.3	6.8 ± 1.2	6.9 ± 1.2	7.9 ± 1.7
	Anabolic	9.3 ± 0.8	10.5 ± 1.1	9.7 ± 1.2	9.1 ± 1.6	11.4 ± 2.8	9.4 ± 1.3	7.8 ± 1.3	11.2 ± 2.1
Semi-T n = 5	Control	8.2 ± 2.7	8.8 ± 2.2	10.9 ± 2.8	10.4 ± 1.5	8.4 ± 1.5	7.0 ± 1.5	12.8 ± 1.6	15.0 ± 2.0
	Anabolic	9.6 ± 2.1	14.4 ± 1.2	15.1 ± 3.2	12.8 ± 1.0	11.5 ± 3.1	9.1 ± 2.4	10.1 ± 1.9	11.7 ± 2.9
$\bar{x}$ n = 17	Control	8.8 ± 1.1	11.8 ± 1.2*	13.3* ± 1.4	11.7 ± 1.0	9.2 ± 1.2	6.9 ± 0.8	9.1 ± 0.9	9.7 ± 1.3
	Anabolic	8.7 ± 0.8	13.1 ± 1.3**	13.5** ± 1.4	10.2 ± 1.3	10.8 ± 1.5	9.4 ± 1.1	8.8 ± 1.1	9.6 ± 1.3

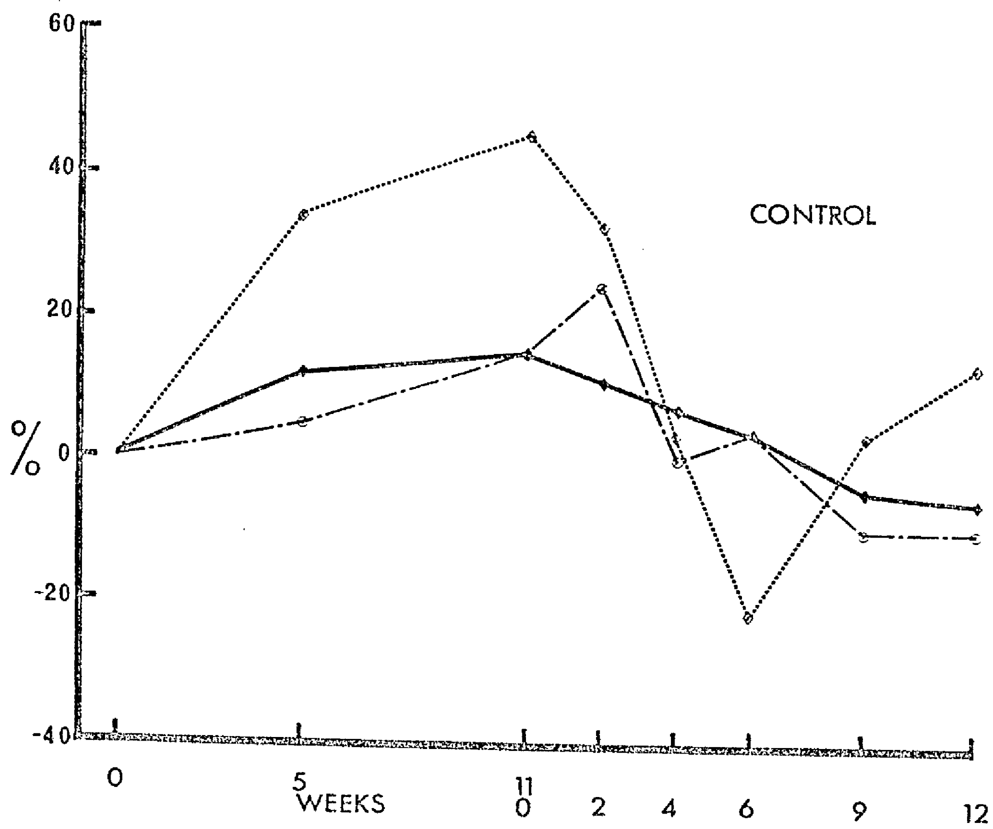
* $P < 0.05$ when compared to pretraining value

** $P < 0.02$ when compared to pretraining value

Figure 4.10 The effect of an anabolic steroid on levels of HAD, CS and Cyt.ox. as aning percentage of pre-training levels. (n = 17) throughout a training and detraining period (n = 6)



Training
Detraining
1
2
60
40
20
0
-20
-40
%
HAD
C S
CYT OX
ANABOLIC



60
40
20
0
-20
-40
%
CONTROL
0
5
WEEKS
11
0
2
4
6
9
12

TABLE 4.15 The effect of an anabolic steroid on the activity of β-Glucuronidase (m.moles substrate hydrolysed/hour/g) throughout a training and detraining period (Mean ± S.E.M.)

Muscle		PT	Post-aerobic	Post-anaerobic	Detraining				
					2	4	6	9	12
MG	Control	1.6 ± 0.4	1.7 ± 0.3	1.7 ± 0.2	2.1 ± 0.4	1.4 ± 0.2	1.4 ± 0.4	1.8 ± 0.4	1.4 ± 0.2
n = 6	Anabolic	1.3 ± 0.1	1.7 ± 0.4	1.9 ± 0.5	2.4** ± 0.2	1.5 ± 0.1	1.3 ± 0.3	1.7 ± 0.3	1.8 ± 0.5
BF	Control	0.9 ± 0.1	1.1 ± 0.3	1.1 ± 0.2	1.7 ± 0.4	1.0 ± 0.2	1.3 ± 0.3	1.4 ± 0.3	1.7 ± 0.5
n = 6	Anabolic	1.1 ± 0.2	2.0 ± 0.6	1.5 ± 0.2	2.8 ± 1.0	1.9 ± 0.8	1.4 ± 0.3	2.3 ± 0.4	2.2 ± 0.9
Semi-T	Control	1.3 ± 0.5	1.2 ± 0.5	0.9 ± 0.2	0.9 ± 0.3	1.3 ± 0.6	1.2 ± 0.3	0.9 ± 0.2	1.2 ± 0.6
n = 5	Anabolic	1.7 ± 0.4	2.0 ± 1.2	1.1 ± 0.4	1.4 ± 0.7	1.4 ± 0.5	1.6 ± 0.6	2.4 ± 1.1	1.3 ± 0.3
$\bar{x}$	Control	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.1	1.6 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.5 ± 0.3
n = 17	Anabolic	1.3 ± 0.1	1.9 ± 0.4	1.5 ± 0.2	2.3 ± 0.4	1.6 ± 0.3	1.4 ± 0.2	2.1 ± 0.3	1.8 ± 0.4

** $P < 0.02$ when compared to pretraining value

Figure 4.11 The effect of an anabolic steroid on levels of β glucuronidase as a percentage of pre-training levels.(n = 17) throughout a training and detraining period (n = 6)

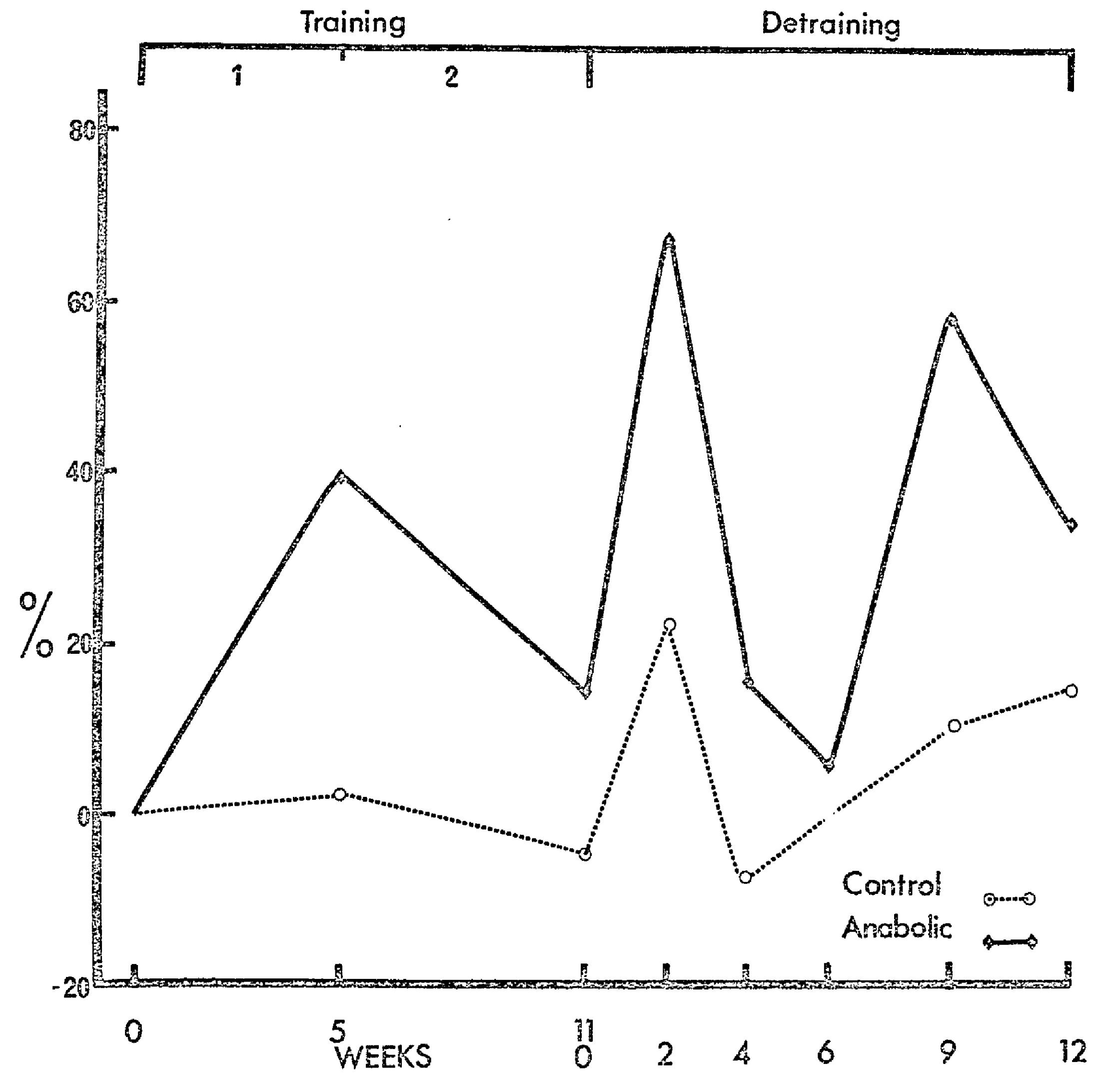

Training
Detraining
1
2
80
60
40
%
20
0
-20
0
5
WEEKS
11
0
2
4
6
9
12
Control
Anabolic

TABLE 4.16 The response, increase (↑), decrease (↓), or no change (-) for an anabolic steroid or placebo administration to 6 horses throughout training

SUMMARY 1

Parameter	Cross-over	Anabolic	Control	Control + Anabolic
Body wt.	1	↓	↓	↓
	2	-	-	-
Chest	1 + 2	-	-	-
Girth	1 + 2	-	-	-
Flank	1	-	-	↓
	2	-	-	-
Stifle →	1	-	-	↑
Stifle	2	-	-	-
Fore limb	1	-	-	↑
	2	-	-	-
Urinary Creatinine	1 + 2	-	-	-
Volume	1 + 2	-	-	-
N_2 excretion	1	↓	-	-
	2	-	-	-

TABLE 4.17 The response, increase (↑), decrease (↓), or no change (-) for an anabolic steroid or placebo administration to 6 horses throughout training

SUMMARY 2

Parameter	Skeletal Muscle	
	Anabolic	Control
% FT MG	↓	↓
BF	-	↓
% FTH MG	↑	↑
BF	-	↑
% ST MG	-	-
BF	-	-
Capillary number	↑	↑
Capillary number/ unit fibre area	-	-
Fibre area	-	-
Fibre ratio HO/LO MG	-	-
BF	↑	-
% area of HO fibres	↑	↑
% area of LO fibres	↓	↓
% H_2O content	-	-
Total protein concentration	-	-
Glycogen concentration MG	↑	↑
BF	↑	↑
LDH	-	-
PFK	-	-
CS	↑	-
HAD	↑	-
Cyt. Ox.	↑	↑
β-Glucuronidase	-	-

DISCUSSION

BODY WEIGHTS, BODY MEASUREMENTS AND URINE ANALYSES

The body weight decrease noted in the first cross-over was not diminished by anabolic steroid administration as there was no difference in response of the anabolic or control group. The decrease was parallelled by a decrease in flank measurement and may have been due to the initial stimulus of training. Weight losses with training have been noted in laboratory animals (Exner *et al*, 1973; Staudte, Exner and Pette, 1973). The failure of the animals to regain weight throughout the detraining period when the flank measurement did increase might be due to redistribution of body composition. The decrease in fore limb measurement suggests atrophy of these muscles and this decrease, with perhaps further skeletal muscle atrophy may offset the increase in flank measurement and thus hold the weight constant.

The changes in body weight which occurred in cross-over 1 but not in cross-over 2 may be because the initial starting weight was less. Why the flank, fore limb and stifle to stifle measurements were not similar in both cross-overs is not clear.

Increases in fore limb measurements have been noted in horses subjected to galloping and treated with nandrolone decanoate (Dietz *et al*, 1973). However there were no controls and the observation in this present study of increases in the control and anabolic groups suggests that the result was an adaptation to gallop training and

not to anabolic steroids. In contrast to this study, Dietz *et al* (1973) found increases in chest measurements and no increase in rump measurement of gallop-trained horses treated with the anabolic steroid. Reasons for the discrepancy between the 2 studies could be due to the longer period of administration (10 months) in Dietz *et al's* study (1973). Also there was no detail of the exact area of measurement and the stifle to stifle measurements in this study may not correspond exactly to those of Dietz's group.

The increase in the urinary nitrogen excretion at the onset of the exercise period can be largely accounted for by the increase in the protein content of the feed. Similarly the increase in urine volume was likely to be a result of the increased dry nature of the supplement causing an increased water intake. The nitrogen excretion result in cross-over 1 suggests that there is some anabolic activity in the treated group. However, the changes in nitrogen excretion were negligible in terms of body weight changes (Discussion, Section 3). The creatinine results are difficult to interpret as, in addition to those effects discussed in Section 3, the muscle was being subjected to various workloads.

Increases in body weight with anabolic steroid administration (Casner *et al*, 1971; Hervey *et al*, 1976) have been attributed to an accumulation of intracellular potassium and water. In the present study and that of Hickson *et al* (1976a) no accumulation of water was noted.

MUSCLE HISTOCHEMISTRY

The increase in the percentage of FTH fibres during training in the MG and the control group of the BF agrees with the results of Guy and Snow (1977a). However the anabolic group of the BF at the post-anaerobic stage was not significantly different from pretraining levels. This apparent effect on the percentage of FTH fibres was similar to that in the resting study. The increase in the HO:LO ratio in the BF of the anabolic group implies an increase in the area of HO fibres relative to the LO fibres. Although the reason for the non-parallel hypertrophy of the fibres is not clear in this study, tentative suggestions may be made. It is possible that the anabolic steroids had a direct effect on the HO fibres, causing them to hypertrophy and consequently during training it would not have been necessary to convert FT to FTH. Alternatively it may be that the HO fibres were forced to hypertrophy because the anabolic steroids had prevented the conversion of FT to FTH. A third possibility is that the hypertrophy was causing a dilution effect of the marker enzyme which counteracted the effect noted with training in the controls.

Transformation of ST to FT has been noted in Thoroughbreds with training (Guy and Snow, 1977a). In this present study there was no alteration in the percentage of total FT or ST fibres in either muscle or when the values for both muscles were combined. Further study would be needed to clarify this point.

FIBRE AREAS

The observation that the HO fibres were smaller than the LO is in accordance with reports in horse (Lindholm, 1974; Snow and Guy, 1980), man (Thorstensson, 1976) and rat (Hickson *et al*, 1976b).

The effects of strength training, in man, have been carried out on sections stained for ATP-ase, and have indicated an increase in the FT:ST ratio (Thorstensson, 1976) and an increase in the IIA:IIB ratio (Costill *et al*, 1979). Although it has been stated (see Section 1) that the Type I and Type II classifications are not directly interchangeable with the metabolic ones used in this study, generally the increase in IIA:IIB ratio is equivalent to an increase in FTH:FT which is comparable with the present findings.

It is surprising to find so few studies investigating the effects of anabolic steroids on muscle fibre area considering the association anabolic steroids have with increases in muscle bulk. No human studies have looked at biopsy material of anabolic-treated subjects. In the rat Hickson *et al* (1976b) found no change in fibre areas either with sprint training alone or with sprint training and the anabolic steroid, dianabol. However the measurements were without subdivision of fibre types and no data was therefore given on ratios. Also there was no control on food intake or any measurement of the adequacy of the diet for animals in training and it may be that in Hickson *et al's* (1976b) study the diet was

not sufficient to allow muscle hypertrophy to occur. In the present study, the almost total excretion of the protein concentrate in the control group is justification for assuming that sufficient protein supplements were given to the horses.

CAPILLARY DENSITY

The observation that capillary number relates to fibre area and that the HO fibres have a greater capillary number/unit fibre area than the LO fibres is in agreement with reports in other species (Romanul, 1965; Rippol, Sillau and Banchero, 1979). When the capillary number was expressed/unit fibre area no increases were noted with training. However, reservation must be attached to these figures as no account was made, in the fibre areas, for possible contraction of the fibres. There are other reports finding no change in capillarity with training (Saltin, Blomquist, Mitchell, Johnston, Wildenthal and Chapman, 1968; Hermansen and Wachtlova, 1971). However the more recent studies report an increase in capillaries/ unit fibre area with endurance training (Carrow, Brown and van Huss, 1967; Andersen, 1975; Andersen and Henriksson, 1977; Ingjer, 1979a). Discrepancies arise from these latter studies. Andersen and Henriksson (1977) found the increase in capillary number to be equal in all fibre types whereas Ingjer (1979a) found the increase to be greater in Type I fibres and Carrow *et al* (1967) in rats found the increase to be greatest in 'white' muscle (Type II) rather than 'red'. These differences could be due to

different training régimes. The endurance training in the study by Ingjer (1979a) was 3 times the duration of the study by Andersen and Henriksson (1977). The study by Carrow *et al* (1967) incorporated strength work in the endurance training programme and this may account for the increased capillarity in the 'white' fibres. Chronic stimulation of rabbit fast muscles at a frequency naturally occurring in nerves to slow muscles (10 HZ) resulted in an increase in capillary density although this was a general response and the capillary density was not related to fibre type (Brown, Cotter, Hudlicka and Vrbova, 1976). In the latter study the changes in capillary density occurred before changes in succinate dehydrogenase activity and it is suggested that the increase in the oxidative capacity of the stimulated muscle might be induced by a better supply of oxygen to the muscle cells during contraction. Studies using training as a stimulus have, however, shown the converse (Andersen and Henriksson, 1977, Muller 1976).

The results, in this present study, that anaerobic work did not produce an increase in capillary density are expected as the function of the capillary to the fibre is principally to enhance its aerobic capacity. The reason for no increase with aerobic training was most likely due to the relatively short exposure to this type of work (5 weeks).

Some of the discrepancies noted in the literature on capillarisation may be due to methodology.

The justification for estimating capillary density on cross-sectioned skeletal muscle has been questioned by Appell and Hammersen (1978) who suggested that increases noted with endurance training may be accounted for by an alteration in capillary pattern, that is one which deviates from the straight and parallel pattern described by Krogh (1918-1919). This would lead to an over-estimation of capillary numbers and an increased capillary count on E/M sections when compared to histochemical sections. This latter point has been reported (Ingjer, 1979b).

BIOCHEMISTRY

The observation that total protein content does not alter with training is in agreement with studies in the horse (Snow and Guy, 1979) and other species (Holloszy, Oscia, Don, Molé, 1970). Although the effects of exercise together with anabolic-steroid administration have not been studied with respect to this parameter, it has been shown that dianabol, given to rats, in conjunction with a surgically-induced hypertrophy does not alter the protein concentration of the skeletal muscle (Ianuzzo and Chen, 1976).

It has been reported that testosterone increases the glycogen content of skeletal muscles (Leonard, 1952; Bergamini, Bombara and Pelligrino, 1969; Bergamini, 1975) and it has also been shown that the glycogen overshoot noted after repeated bouts of strenuous exercise (Bergström and Hultman, 1966) may be partly dependent on testosterone

(Gillespie and Edgerton, 1970). Although increases in glycogen concentration have been noted with sprint exercise in rats (Hickson *et al*, 1976b) no additional effect was noted in a trained group treated with dianabol. In this present study an increase in glycogen concentration was noted in all muscles with training except the BF muscle of the anabolic group. However, the discrepancy of this group was due only to 1 animal and therefore there is no justification for assuming this to be a notable difference. The present results are therefore in agreement with the reports of increases in glycogen concentration in the horse with training (Guy and Snow, 1977a). Also the lack of any additional effect of anabolic steroids on the increase noted with training is in agreement with studies in rats (Hickson *et al*, 1976b).

The effects of sprint training on skeletal muscle enzyme activities have produced varying reports. One of the main reasons for this is the different exercise programmes involved. To be a true sprint programme involving no endurance the frequency and duration of the bouts of exercise must be limited. In view of the great reliance on anaerobic metabolism during short-term maximal work it might be expected that the adaptations would be different from those of endurance training. The activity of the glycolytic system represented in this study by LDH and PFK was unaltered during training. Similar studies in various species have produced differing results with reference to these enzymes. No change in

PFK activity was noted in strength trained humans (Thorstensson, Hulten, Dobeln and Karlsson, 1976) although Costill *et al* (1979) have shown increases in PFK activity with strength training. However, the Costill *et al* (1979) paper illustrates the specificity of the exercise in producing enzyme activity increases. Two bouts of 30 second work produced increases in PFK, creatine phosphokinase (CPK), myokinase (MK), malate dehydrogenase (MDH) and SDH whereas a similar work duration divided into 6 second bouts only showed increases in PFK activity. LDH activity with repetitive sprint training has shown increases (Guy and Snow, 1977a), decreases (Hickson, Heusner and van Huss, 1975) and no change (Exner *et al*, 1973; Hickson *et al*, 1976b; Costill *et al*, 1979). The effect of an anabolic steroid in conjunction with exercise has been studied only in rats which were isometrically trained (Exner *et al*, 1973) and the exercise programme involved a large endurance factor. However, there were no greater effects on muscle enzymes in the male rats treated with anabolics when compared to exercising controls.

The reason for the increase in activity during detraining in the anabolic groups in the present study at 4 weeks as compared to 2 weeks is not clear. However the progressive decrease thereafter may be related to the 'wearing off' effect of the steroid noted in earlier studies by reversion of nitrogen retention (Kochakian, 1935) and more recently noted by athletes themselves (Freed *et al*, 1976). However, there was no significant difference

between control and anabolic-treated animals at the 12 week detraining sample.

The increase in CS activity was only significant in the anabolic steroid group although it did increase in controls. Other studies of increases in this enzyme's activity with sprint training have been reported (Staudte *et al*, 1973; Exner *et al*, 1973; Guy and Snow, 1977a). The increase in this study was extremely small in comparison to that of Guy and Snow (1977a). However, it has been pointed out that differences arise according to whether aerobic or anaerobic metabolism was primarily involved and the extent to which the enzyme activities change is proportional to the intensity of exercise (Fitts, Booth, Winder and Holloszy, 1975). In the study by Guy and Snow (1977a) the repetitive sprint régime comprised a large endurance factor.

HAD responded similarly to CS during training. In the training régime used by Straub *et al* (1975) the activity of this enzyme did not alter, although increases were noted in the study by Guy and Snow (1977a). The study of Straub *et al* (1975) was on small numbers and the greater variation found in this enzyme's activity may have masked any significant changes. The enhancement of HAD activity would increase the ability of muscle to metabolise fats as an energy source. The increased oxidation of fat would lead to increased citrate levels which would inhibit PFK (Hoffman, 1976). PFK is thought to be the regulatory enzyme of glycolysis (Review: Newsholme and

Start, 1973), and will therefore modulate LDH activity. This may be one of the reasons that the activity of the glycolytic enzymes did not increase in this study although LDH activity did increase in the presence of increased HAD activity in the study of Guy and Snow (1977a, 1979).

Disproportionate activities of the TCA cycle enzymes with HAD have been noted in single fibre analysis of FT fibres (Spamer and Pette, 1979). As the predominant fibre type in the muscles presently studied is fast twitch, then the difference in CS and HAD activity over the detraining period is not unusual.

Cyt. ox. was the only enzyme to show significantly increased activity after aerobic training. The difference was significant in both groups although the anabolic group did show a greater response. Increases have been noted with endurance exercise where they may be contributing to a shift towards a greater fat relative to carbohydrate metabolism (Baldwin, Klinkerpuss, Terjung, Molé and Holloszy, 1972; Henriksson and Reitman, 1976). The necessity for such an enhancement in sprint exercise is not clear.

β-Glucuronidase did not alter throughout training. This enzyme was of particular interest as it has been noted to be related to atrophy of skeletal muscle (Weinstock and Iodice, 1969) and also to increase its activity after exhaustive exercise (Pilstrom, Vihko, Aström and Årstila, 1978; Vihko, Salminen and Rantamaki, 1978; Vihko *et al*, 1979). The time course of the activity changes closely resembled that earlier found to be caused by ischaemia in

rat muscles (Shannon, Adams and Courtice, 1974). It had been suggested that the 2 treatments, exhaustive exercise and temporary ischaemia, cause similar cell injuries and that the lysosomal system involved was also similar in the post-stress recovery of the fibres from these injuries (Vihko *et al*, 1978). The absence of such an effect in this study may be due to the fact that the effect was not noted in trained animals (Schott and Terjung, 1979).

In summary the enzyme results showed a trend towards an increase in activity with training although the increases were significant only in the anabolic-treated group. The exception was Cyt. ox. which increased its activity in both the anabolic and control group with training. An increase in enzyme activity has been noted in resting female rats treated with nandrolone decanoate although it was not improved upon by training (Exner *et al*, 1973).

The enzyme response was considerably less than that noted by Guy and Snow (1977a) and may be due to the different training régimes as this would appear to be critical (Costill *et al*, 1979). The lack of any 'rebound' effect in the detraining period may be due to the fact that minimal changes occurred within the training period.

SECTION 5

THE EFFECTS OF ACUTE EXERCISE ON SOME ASPECTS OF SKELETAL MUSCLE MORPHOLOGY AND METABOLISM

The metabolic and morphological changes associated with acute exercise are still not well understood. The factors believed to cause fatigue are many and varied. In this part of the present study some aspects of fatigue were investigated. An electron microscopical investigation was carried out on skeletal muscle samples taken before and after exercise. In addition, on similar samples, the concentrations of glycogen, lactate and pyruvate were assayed.

ELECTRON MICROSCOPY STUDY

An increase in volume, occurring in muscle exposed to some sort of stimulus, is not a new observation. Hill and Kupalov (1930) described swelling of muscle as a result of acute exercise. They attributed the swelling to an increase in osmotic pressure, caused by the accumulation of lactate and creatine within the cells. Although these metabolites may enhance the uptake of extracellular fluid into the cells, Hill himself was aware that the osmotic pressure needed to explain the swellings could not be totally accounted for by metabolites known to be produced. Mitochondrial swellings produced by exhaustive endurance exercise have also been reported. Most of the work originates from the investigation of Gollnick and King (1969). They found that skeletal muscle mitochondria of rats after exercise were greatly enlarged, producing large pale matrices with sparse and disorientated cristae. They qualified this by noting that the swellings only occurred

in the trained animals and only when the exercise was of a running nature.

Since that report the effect of endurance exercise on muscle mitochondria has produced conflicting evidence in the literature. Ultrastructural studies supporting Gollnick and King's (1969) view are evident (Gale, 1974; Bowers, Hubbard, Smoake, Dawn and Nilson, 1974) and there is biochemical evidence indicating that exhaustion of trained rats causes a decrease in the capacity of skeletal muscle to oxidize important energy fuels (Dohm, Huston, Askew and Weiser, 1972). In contrast, Terjung, Baldwin, Molé, Klinkerfuss and Holloszy (1972) have combined biochemical and electron microscopical examination of skeletal and heart muscle and have found no disruption of the mitochondria. Differences arising purely from the various fixatives used do not explain fully the anomolies (Gale, 1974).

An ultrastructural study of equine skeletal muscle has been carried out on clinical cases in Standardbred horses (Lindholm, Johansson and Kjaersgaard, 1974a). The animals studied showed symptoms of 'tying up' or 'equine rhabdomyolysis', a myopathy common in both Standardbreds and Thoroughbreds manifested even after light exercise.

The present exercise study on horses was carried out on clinically normal animals and was undertaken in order to gain more insight into the effects of exercise on skeletal muscle ultrastructure. In addition, with this species, it was possible to extend the previous studies

beyond that of the effects of endurance exercise and incorporate a study on the effects of strength exercise on skeletal muscle.

MATERIALS AND METHODS 1

ANIMALS

To determine normal ultrastructure of equine skeletal muscle and the influence of different fixation procedures, muscle samples from 14 clinically normal resting horses were obtained by biopsy. A further 4 samples were obtained from animals post-mortem. All of the animals had, as far as could be determined, no illnesses directly connected with their musculo-skeletal system. Five horses (4 Thoroughbreds and 1 Heavy Hunter) were then trained and tested using two different exercise régimes. The training programme consisted of 4 days submaximal work and 2 days of sprinting three times over 600 m. The training programme lasted 10 weeks. A further period of 5 weeks of sprinting over various distances was also included for 3 Thoroughbreds.

The strength exercise trials were carried out on the 3 Thoroughbreds during the 5 weeks of sprinting. Each horse was galloped flat out over a distance of 500 m. four times in succession with an interval of 5 minutes between each gallop.

The endurance exercise trials were carried out during the 10 week training period. Four trials consisted of a run of 22.4 Km. and a further 4 trials consisted of a 15 Km. ride in an indoor riding school.

Both of these exercise régimes worked the horses to near their limits of performance. During the training period the horses were housed in loose boxes and maintained

on a constant diet throughout the experiments.

BIOPSY SAMPLING

The needle biopsy technique was used as described on live animals and the biopsy clamp was used on animals post-mortem (Section 2).

Samples were taken on two occasions from each distance once at the onset of the training programme and then again towards the end of the training programme.

The main site used for biopsy was the MG. Occasionally the deltoid, vastus lateralis and semi-T were also sampled in the resting animals. For comparative purposes the semi-T was sampled on one exercise occasion as well as the MG.

Pre- and post-exercise samples were taken about 2 cm. apart to avoid sampling any damaged tissue and the post-exercise samples were taken immediately after completion of the exercise.

METHODS OF FIXATION

The fixatives used in this study were of 2 basic types.

1. 1% Osmium tetroxide (OsO_4) (S-collidine buffer)

Reagents

1) 2% OsO_4 in distilled water

2) Buffer:

S-collidine	2.67 ml
Dist H_2O	50 ml
1M HCl	9.0 ml
Dist H_2O up to	100 ml

The final concentration of buffer is 0.2M at pH 7.4 → 7.5.

The osmium tetroxide fixative is made up just prior to use.

1 part buffer to 2 parts 2% osmium tetroxide.
The final concentrations are therefore 1.33% osmium tetroxide in 0.067M buffer (osmolarity of vehicle = 142 milliosmoles).

The tissue was fixed for 1 hour. This was largely empirically determined. It is essentially a compromise between the need for complete penetration of the fixative into the block and the fact that protein and other substances are progressively leached out when they are immersed in osmium fixative. Figures of 0.75 mm/hr have been published for the penetration rate of osmium tetroxide solutions (Burkl and Schiechl, 1968) and these agree with the rate reported by Hägström and Bohr (1960) for 1% OsO_4 in distilled H_2O.

Although osmium is generally accepted to have a slower penetration rate than the aldehydes, there is evidence that in small blocks glutaraldehyde is, in fact, slower (Ericsson and Biberfield, 1967). However, for optimum preservation throughout the block with osmium, the block must not exceed 0.5 mm^3. Great care was therefore taken with these samples to make sure that the blocks were less than the critical size.

Because of the importance of the speed of the fixative penetration, especially in a possible dynamic situation such as exercise, some blocks were also fixed in a Modified Karnovsky's (Karnovsky, 1965).

2. Modified Karnovsky's

Reagents

1) 0.2M Buffer 50 ml

2) 10% Paraformaldehyde 20 ml

3) 25% Glutaraldehyde 10 ml

4) Dist H_2O up to 100 ml

This gives 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M buffer.

The combination of paraformaldehyde and glutaraldehyde has both the properties of being rapidly penetrating (the formaldehyde component) and of establishing firm cross-linking of the proteins (the glutaraldehyde component).

The efficiency of the aldehydes as cross-linking agents for protein is counteracted by their inability to stabilise unsaturated lipids or phospholipids and it has been shown in smooth muscle (Jones, Somlyo and Somlyo, 1973) that cells can still act as osmometers after glutaraldehyde fixation. It was necessary therefore to post fix the samples in 1% osmium tetroxide for 1 hour.

CALCIUM

The addition of calcium to fixation media is normally carried out to aid the preservation of membrane structure. 1mM was added to some fixatives and compared to controls.

FIXATION TIMES AND TEMPERATURE

Fixation times, in Karnovsky's, of 3, 6 and 24 hours were experimented with and some samples were kept at $0^{o}C$ throughout the complete fixation procedure.

OSMOLARITY

The fixative medium may precede that of the

fixative molecules (Palade, 1952) in the case of osmium. The cell constituents are therefore liable to damage from the medium. In order to allow for this some samples were fixed in 1% osmium fixative made up with saline (osmolarity of vehicle=300 mosmoles) (Knauer Electronic Semi-Micro Osmometer).

Preservation of the cell constituents may be aided by the addition of sucrose (Caulfield, 1957). Some of the fixation media therefore included a 7.5% sucrose component (osmolarity of vehicle = 377 milliosmoles).

BUFFER

Two buffers were used in conjunction with the Modified Karnovsky's fixative. These were cacodylate (osmolarity of vehicle = 191 milliosmoles) and phosphate (osmolarity of vehicle = 169 milliosmoles).

PROCESSING

In all the given fixations, the tissues were dehydrated through graded acetone and cleared in propylene oxide for 40 minutes. They were then left in a 50:50 propylene oxide/Spurr epoxy resin mixture for 1 hour and were transferred to fresh 100% Spurr resin and left overnight. The tissues were then flat embedded and put in a 60°C oven for 14 hours.

The blocks were trimmed on an LKB pyramitome and 600 Å sections were cut using glass knives on an LKB Mark III ultratome.

Longitudinal sections were mounted on copper grids, stained in alcoholic uranyl-acetate followed by lead citrate

(Reynolds, 1963) and photographed in an Hitachi HS8 electron microscope at an accelerating voltage of 50 Kv. Electron micrographs were taken on Ilford SP332 photographic film.

RESULTS 1

RESTING

The needle biopsy technique contracts the muscle (Figure 5.1) resulting in a decrease or complete absence of any I band. In order to determine whether the method itself was causing any disruption of the organelles a biopsy clamp was used to obtain muscle from a post-mortem horse (Figure 5.2). The muscle was held extended throughout the fixation and processing period using this technique. There was no evidence, in comparing micrographs of the two methods, to suggest that the needle biopsy altered the non-myofibrillar ultrastructure of skeletal muscle.

All muscle tissue taken from resting animals whether fixed in osmium or Modified Karnovsky's showed similar morphology to that of other species. Mitochondria fixed in the standard aldehyde had the densest matrices and the cristae were consequently least defined. The cacodylate-buffered variant of Karnovsky's fixative produced less dense matrices than those seen with phosphate buffer but not as pale as those produced by osmium fixation. The mitochondria of the osmium plus sucrose samples was, perhaps, slightly more dense than the other osmium fixatives although this was not always evident.

There was no mitochondrial disruption either deep in the fibre or in the subsarcolemmal regions with any of the fixatives employed.

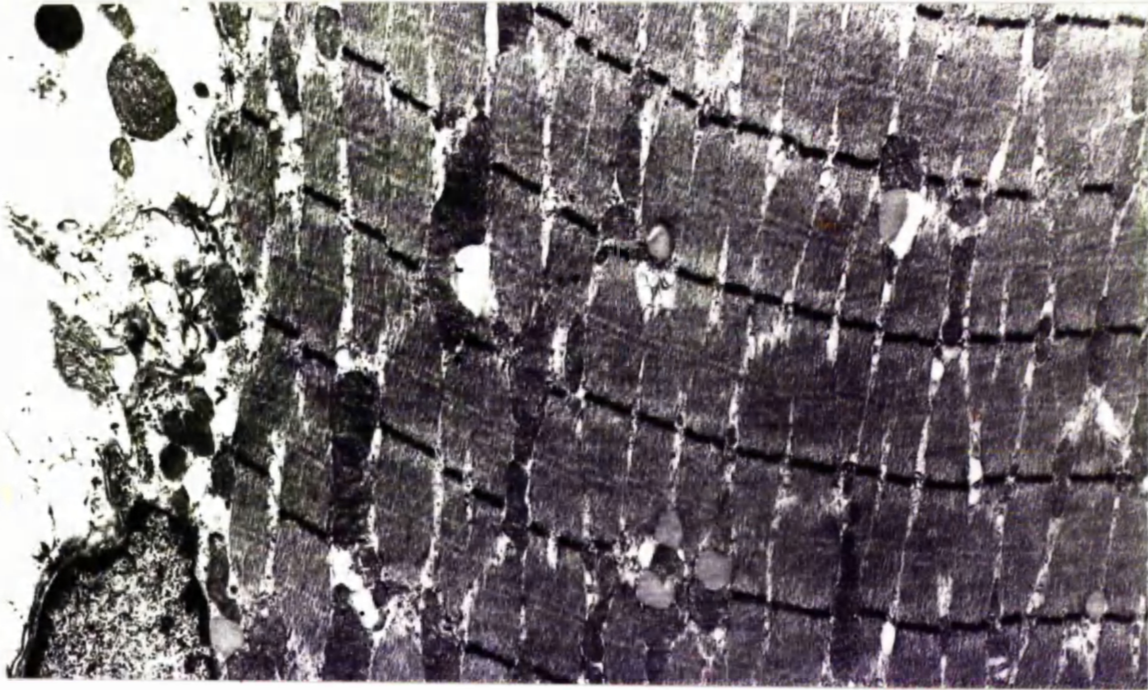


Figure 5.1 Skeletal muscle taken from a resting horse

Fixative : Modified Karnovsky

Buffer : Phosphate

Mag. : 8.4×10^3

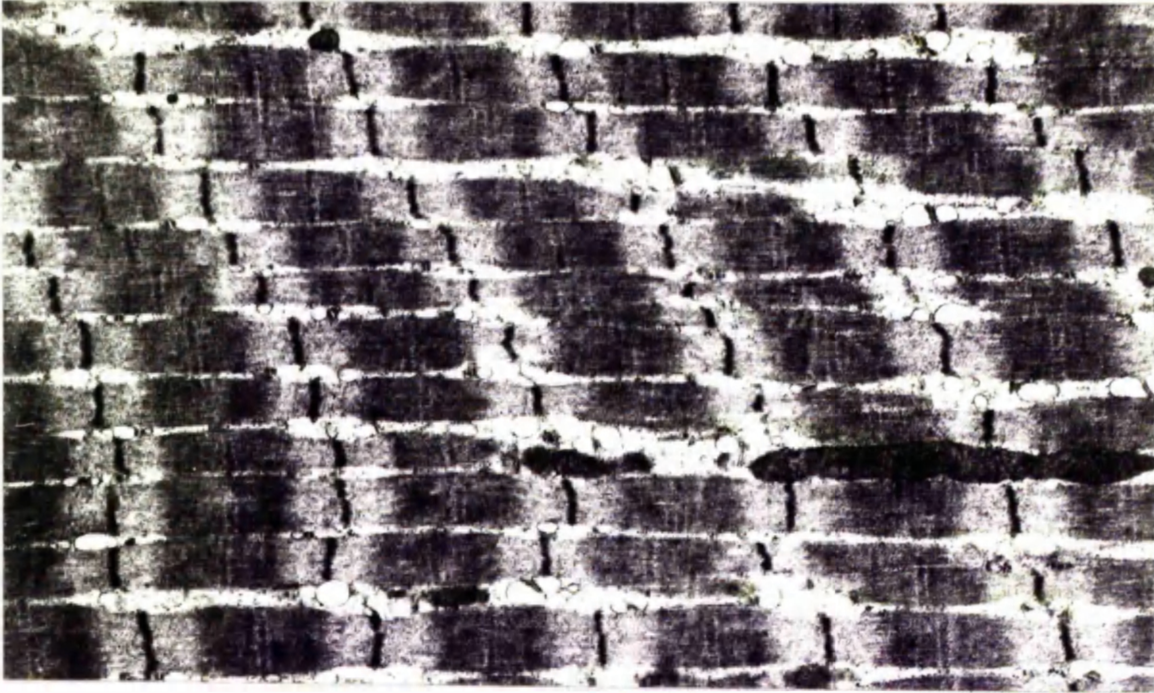


Figure 5.2 Skeletal muscle, obtained by biopsy clamp from a post-mortem horse

Fixative : Modified Karnovsky

Buffer : Phosphate

Mag. : 12×10^3

STRENGTH EXERCISE

All the post-exercise samples showed mitochondrial disruption whereas there was none in the controls (Table 5.1; Figure 5.3). The swelling of the mitochondria in the post-exercise samples was verified by planimeter measurement of the photomicrographs. Paired pre- and post-exercise samples fixed in osmium with s-collidine buffer, were measured for mitochondrial area. Values for the pre-exercise samples of the 3 Thoroughbreds gave a mean value of 1.30 ± 0.03 μm^2 (n = 400) and 2.06 ± 0.06 μm^2 (n = 340) for the post-exercise samples. This difference was statistically significant ($p < 0.02$).

The cristae appeared sparse and disorientated and there were areas where there was no sign of an outer mitochondrial envelope for a substantial part of the organelle. This disruption was not uniform throughout the fibre (Figure 5.4). In general, disruption of the subsarcolemmal mitochondria was greater than those deep in the fibre. The sarcolemma, nuclei and myofibrils were indistinguishable from those of controls. In some cases, however, sarcoplasmic reticulum swelling was present.

It was also noted that large stores of glycogen were still present in the post-exercise samples (Figure 5.5). This plate also demonstrates the failure of osmium to preserve nuclear morphology.

CALCIUM

The addition of 1mM Ca^{2+} to the fixative did not appear to influence the degree of swelling (Table 5.1).

TABLE 5.1 The effect of strength exercise on skeletal muscle mitochondria prepared in a variety of fixatives

Horse	Muscle site	Fixative	Buffer	Swollen mitochondria
Thoroughbred 1 (1)	M.G.	P/G + Ca^{2+}	Phosphate	+
	Semi-T	P/G + Ca^{2+}	Phosphate	+
(2)	M.G.	P/G $4^{o}C$	Cacodylate	++
	M.G.	P/G Room temp.	Cacodylate	++
	M.G.	P/G	Phosphate	+
Thoroughbred 2 (1)	M.G.	P/G 3 hrs	Phosphate	+
		P/G 6 hrs	Phosphate	+
		P/G 24 hrs	Phosphate	+
(2)	M.G.	P/G + Ca^{2+}	Phosphate	+
	M.G.	P/G	Phosphate	+
	M.G.	Osmium	S-collidine	++
Thoroughbred 3 (1)	M.G.	P/G	Cacodylate	++
	M.G.	Osmium	S-collidine	++

M.G. Middle gluteal
Semi-T Semitendinosus
\+ Indicates presence and degree of swelling.

(1) Trial near onset of training period
(2) Trial after a period of training
P/G Modified Karnovsky's.

Figure 5.3 Skeletal muscle, obtained after strength exercise

Fixative : Osmium

Buffer : s-collidine

Mag. : 8.4×10^3

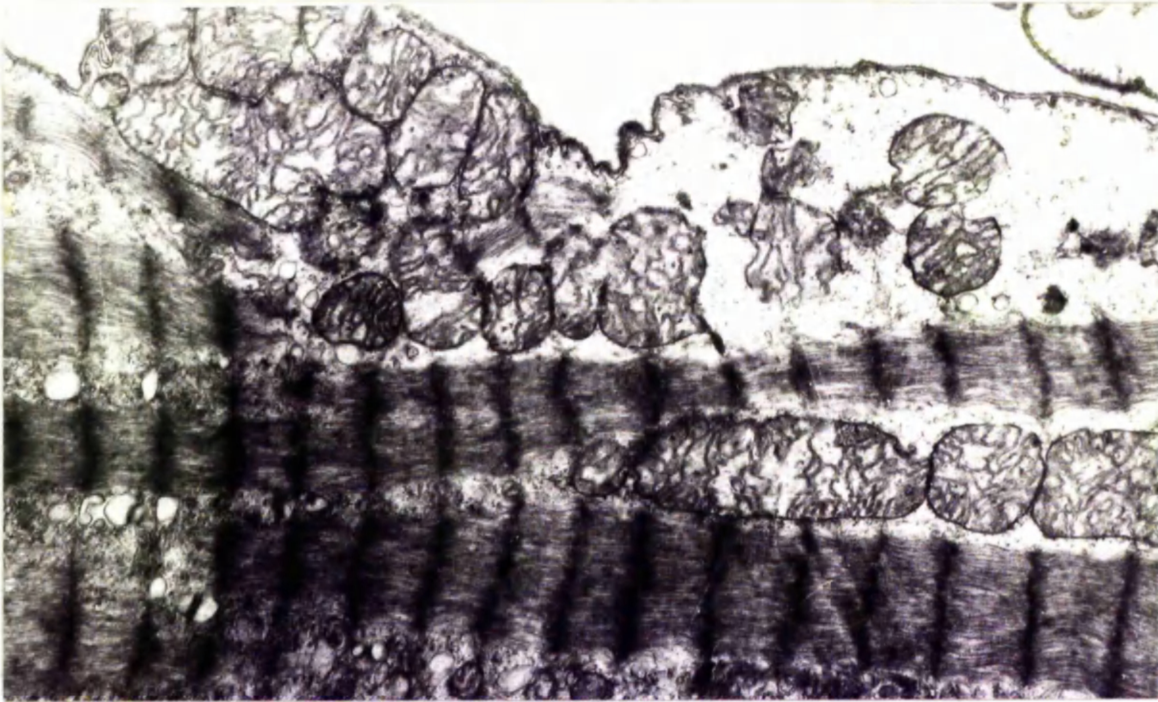


Figure 5.4 Skeletal muscle obtained after strength exercise showing non-uniformity of mitochondrial disruption

Fixative : Osmium

Buffer : s-collidine

Mag. : 13×10^3

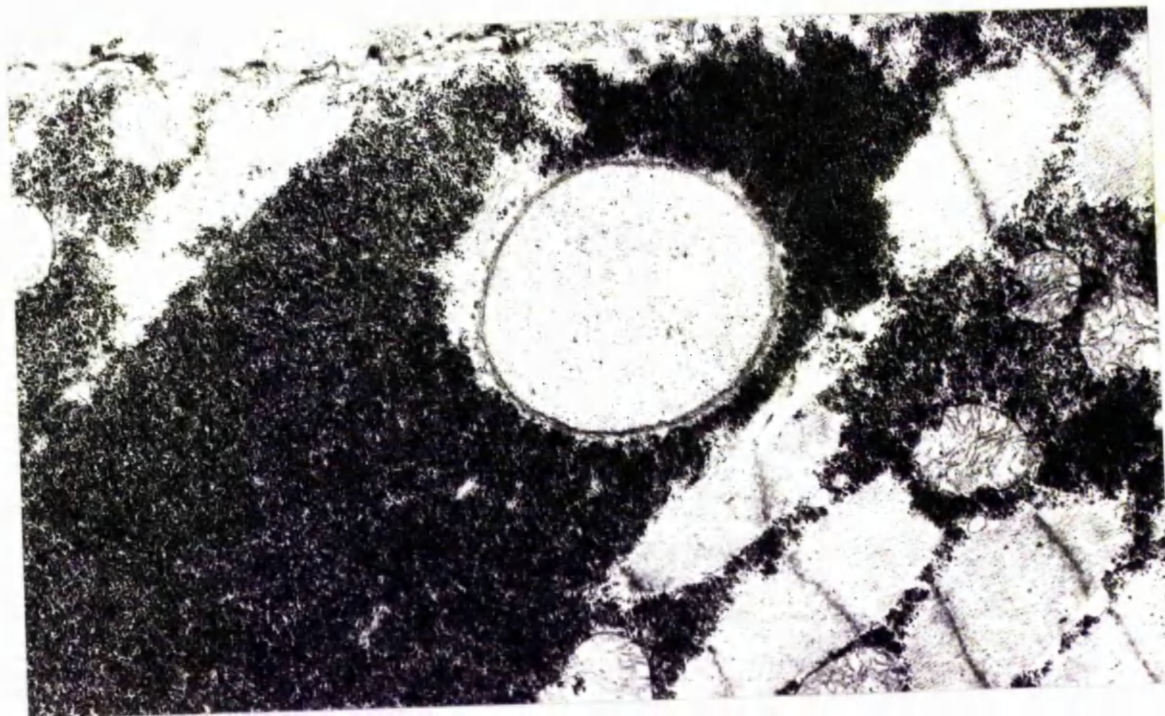


Figure 5.5 Skeletal muscle sample obtained after strength exercise showing abundant muscle glycogen

Fixative : Osmium

Buffer : s-collidine

Mag. : 12×10^3

FIXATION TIMES AND TEMPERATURE

Samples processed at $4^{o}C$ were no different from those processed at room temperature, nor were there any differences between fixation times of 3, 6 and 24 hours (Table 5.1).

BUFFER

Cacodylate-buffered aldehyde fixative specimens showed greater swellings than those seen with phosphate.

MUSCLE SELECTION

Disruption of the semi-T muscle was not detectably different from that of the MG.

ENDURANCE EXERCISE

Swelling of the mitochondria was never noted in the resting samples but did occur in some samples after endurance exercise (Table 5.2). Planimeter measurement of mitochondria of paired pre- and post-exercise samples of the 4 Thoroughbreds, fixed in osmium with s-collidine buffer, gave mean values of $1.30 \pm 0.04\ \mu m^2$ (n = 360) and $2.00 \pm 0.08\ \mu m^2$ (n = 326). This difference was statistically significant ($p < 0.02$).

The post-exercise values of the endurance test did not differ significantly from those of the strength test.

Samples fixed in 1% osmium tetroxide displayed obvious rupturing of some outer mitochondrial membranes in subsarcolemmal sites (Figure 5.6). This was never seen deep in the fibre or after aldehyde fixation.

Another phenomenon noticed

was occasional fibrillar splitting (Figure 5.7a, b). This was noted in both pre- and post-exercise

TABLE 5.2 The effect of endurance exercise on skeletal muscle mitochondria, prepared in a variety of fixatives

Horse	Muscle site	Fixative	Buffer	Swollen mitochondria
Thoroughbred 1 (1)	M.G.	P/G + Ca^{2+}	Phosphate	None
		Osmium + sucrose	S-collidine	None
(2)	M.G.	Osmium	S-collidine	++
		Osmium	S-collidine(saline)	++
Thoroughbred 2 (1)	M.G.	P/G + Ca^{2+}	Phosphate	None
		Osmium + sucrose	S-collidine	None
(2)	M.G.	Osmium	S-collidine	++
Thoroughbred 3 (1)	M.G.	P/G	Cacodylate	+
		Osmium + sucrose	S-collidine	None
		Osmium	S-collidine	+++
(2)	M.G.	Osmium	S-collidine	++
Thoroughbred 4 (1)	M.G.	Osmium	S-collidine	++
Heavy Hunter (2)	M.G.	P/G	Cacodylate	None
		P/G	Phosphate	None
		Osmium	S-collidine	None

M.G. Middle gluteal

P/G Modified Karnovsky's

* Indicates presence of swelling.

(1) Trial at onset of training period

(2) Trial after a period of training.

Figure 5.6 Skeletal muscle obtained after endurance exercise

Fixative : Osmium

Buffer : [illegible]-collidine

Mag. : [illegible] $\times 10^3$

Figure 5.7 a) Resting skeletal muscle

Fixative : Modified Karnovsky

Buffer : Phosphate

Mag : 8.4×10^3

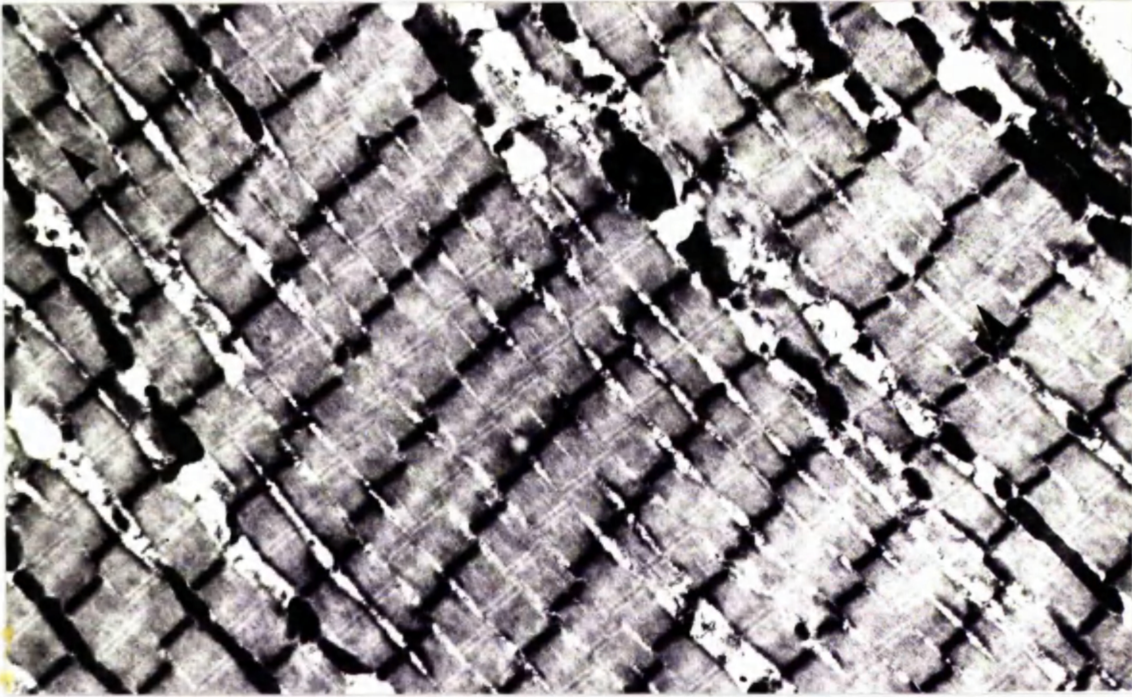
b) Skeletal muscle after endurance exercise

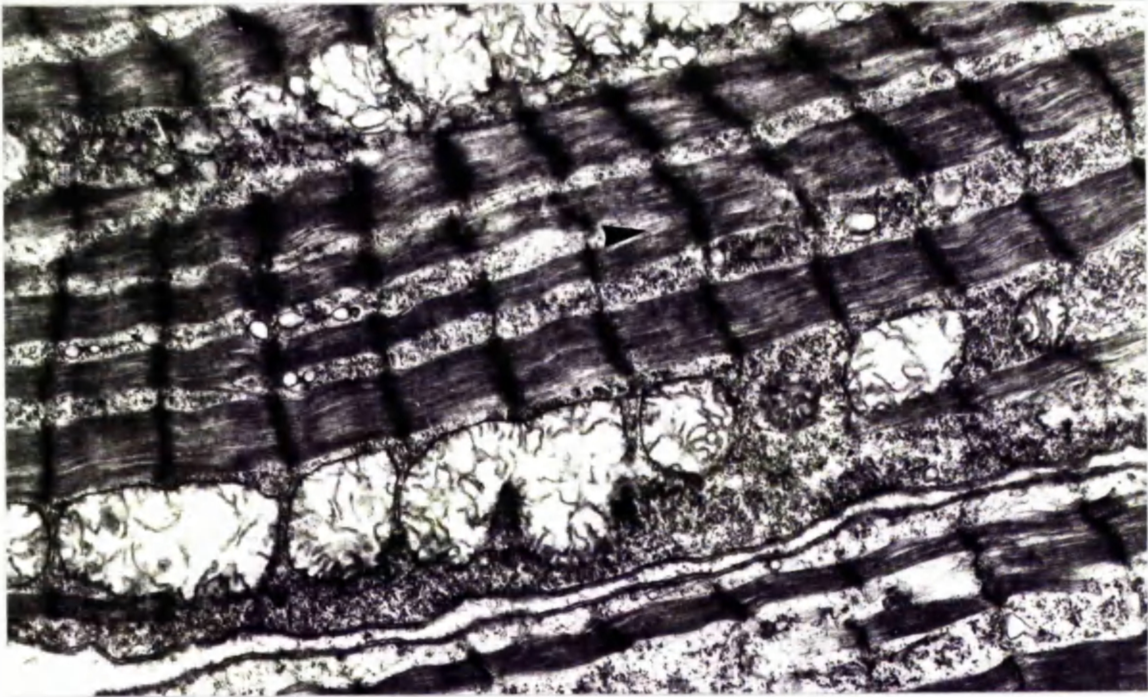
Fixative : Osmium

Buffer : s-collidine

Mag : 12×10^3

→ indicates myofibrillar splitting.





samples.

The Heavy Hunter was the only animal to show no mitochondrial swelling even in specimens fixed in ways which successfully preserved swellings in other submaximally-exercised animals.

OSMOLARITY

The 3 samples fixed in osmium plus 7.5% sucrose showed no swellings. When the osmium was made up to be isosmotic with saline the same kind of swellings were seen as in specimens fixed in the standard osmium fixative.

BUFFER

Samples prepared in phosphate-buffered aldehyde did not show any disruption. It will be noted that although the modified osmium fixatives had not been tried with specimens from strength-exercised animals, the phosphate-buffered aldehyde had yielded positive results in such instances.

One argument against using osmium in this type of experiment has been that the disruption seen might be due to autolysis of the tissue (contrary to views of those such as Ericsson and Biberfield, 1967) if the fixative has not penetrated quickly enough (Gale, 1974; Terjung *et al*, 1972). Although this seems an unlikely complication with specimens as small as those used here, a check was performed. Immersion of 1 sample in Modified Karnovsky's fixative was delayed deliberately for 6 minutes allowing autolysis to occur (Figure 5.8). It is evident that the disruption caused by autolysis is very different from the exercise-induced

swellings. It is therefore concluded that the swellings reported in this study are not due to slow penetration of the fixative.

Figure 5.8 Skeletal muscle, showing imperfect fixative penetration

Fixative : Modified Karnovsky

Buffer : Phosphate

Mag. : 30×10^3

DISCUSSION 1

Although the observations in this study were limited due to the small sample number, the study permits some clarification of a number of points made in the different approaches to the study of ultrastructural disruption caused by exercise.

The results, in general, support those of Gollnick and King (1969) although their finding of mitochondrial disruption being greatest deep in the fibre contrasts with the present study in which disruption was greatest at the sarcolemma. However, studies in ischaemic skeletal muscle have also shown mitochondrial disruption to be concentrated at the periphery (Hanzlikova and Schiaffino, 1977). It is not clear whether the lability of the mitochondria, seen as swelling, was caused by different conditions in peripheral as opposed to central areas of the fibre or by a heterogeneity of the mitochondria themselves.

Disruption was noted after both the strength and endurance exercise, although after endurance exercise the disruption did not occur in all the cases. Mitochondrial integrity was consistently maintained in the Heavy Hunter. This animal showed no mitochondrial disruption even when the fixative was osmium and s-collidine buffer. This observation may be analogous to the untrained animal (Gollnick and King, 1969) where, too, there was no disruption. During the trial the Heavy Hunter stopped on a few occasions and had to be encouraged to go on. This was in contrast

to the Thoroughbreds who although fatigued were still prepared to work on. It is possible therefore that the lack of any mitochondrial disruption in the Heavy Hunter was owing to the fact that the animal's muscle was not sufficiently fatigued. Against this hypothesis, however, there was no difference found in the Thoroughbreds when they were tested at various fitness levels, that is, near the beginning of their training as compared to the trial at the end of a training period. Further work would have to be carried out in order to clarify this point.

Although, in some of the already published literature, the reason given for any disruption in exhausted animals has been attributed to fixation artefact (Terjung *et al*, 1972; Bowers *et al*, 1974), it should be emphasised that in the present study, biopsies taken from resting animals did not show mitochondrial disruption in any of the fixation media studied. This would indicate that although the *in vivo* state may not be indicated by the electron microscope, the sensitivity of the mitochondria to the fixation medium was significantly altered by exhaustive exercise.

By varying the constituents of the fixation medium, it was possible to draw some idea of the nature of the mitochondrial change. In previous ultrastructural studies on exercise (Bowers *et al*, 1974; Gale, 1974), the aldehyde fixative used was glutaraldehyde. This was probably neither 'fast' nor 'strong' enough a fixative to preserve dynamic changes (Jones *et al*, 1973). The difference noted in this study may be due to the inclusion of the faster

penetrating paraformaldehyde component.

The reason for the slight difference in the cacodylate and the phosphate buffer results may be due to the fact that cacodylate is a large anion (impermeant) and will lead to 'chloride withdrawal' from the cell without simultaneous replacement. The resultant unbalanced outflow of negative charge will cause the muscle to depolarize to an extent which in the absence of a 'strong' fixative activates 'chloride withdrawal' contractures (Falk and Lauda, 1959; Faulkes, Pacey and Perry, 1965). This might, in the present experiment, be one reason for the greater dilation of the mitochondria in the cacodylate-buffered samples. Although phosphate also has an extremely low permeability through muscle membrane it does penetrate relatively freely through mitochondrial outer membranes (Jennings, Moore, Shen and Herdson, 1970). Shen and Jennings (1972) have shown that under certain conditions mitochondria will accumulate calcium phosphate *in vivo* and it may be that the phosphate of the fixation medium was involved in preventing or reversing the mitochondrial swellings. Terjung *et al* (1972) denied any evidence for mitochondrial swelling although their micrographs do show some swelling of osmium-fixed tissue (Gale, 1974). The osmium of Terjung *et al's* (1972) samples was buffered in phosphate and it may be that this was the reason for the relatively smaller swellings.

Similarly, it could be argued that the addition of calcium to the fixation medium should have some effect

on mitochondrial disruption. Rossi and Lehninger (1964), in liver, Shen and Jennings (1972), in myocardium and Tate, Bonner and Leslie (1978), in skeletal muscle, have described conditions in which mitochondria will accumulate calcium if phosphate is not present. This cation in excess leads to the uncoupling of oxidative phosphorylation (Potter, Siekevitz and Simonson, 1953), and depression of the TCA cycle by inhibition of isocitrate dehydrogenase (Zammit and Newsholme, 1976). The uncoupling of oxidative phosphorylation was associated with swollen mitochondria (Shen and Jennings, 1972). Although the addition of extracellular calcium to the fixation medium showed disruption of exhausted muscle mitochondria where it had not been previously noted (Bowers *et al*, 1974) there was no indication of an effect in the present study. However as Bowers *et al* (1974) do not quote a figure for the amount of calcium added and the degree of mitochondrial swelling is dependent upon the Ca^{2+} concentration (Rossi and Lehninger, 1964) a difference in calcium concentrations in the two studies is the most likely reason for the contradictory results.

Until recently it was common practice to add sucrose to an osmium fixative. However, it is now widely accepted that the osmolarity of the fixative vehicle should be 60% of that normally surrounding the tissue (Denton and Bone, 1971; Jones *et al*, 1973). Although this figure varies in different tissues, approximations may be made. The standard cacodylate and phosphate-buffered Karnovsky's and the s-collidine-buffered osmium fall into the 60% range.

However, on the addition of 7.5% sucrose, the medium becomes hyperosmolar and would tend to reverse or prevent the mitochondrial swelling. This difference in osmolarity may explain the lack of response in the tissues prepared by Bowers *et al* (1974) where sucrose was added to the glutaraldehyde fixative. The osmium fixative made up in saline in the present study should theoretically have shown less swelling than the standard preparation; however, no clear distinction could be made. The osmolarity of the fixative vehicle is therefore of prime importance when considering these ultrastructural changes.

As no swellings were noted in the resting state and the osmolarity of the fixative vehicle does not alter with the pre- and post-exercise samples, then it must be changes occurring in the mitochondria. The most likely reason for the changes is the accumulation of calcium by the mitochondria (Tate et al 1978). This may be a result of a failure of the SR, during exercise, to restore resting myoplasmic calcium levels. In addition, during strength exercise, the membrane permeability of the organelles may be altered by a lowering of pH.

Differences between different types of exercise have been noted with reference to mitochondrial calcium uptake (Bonner, Leslie, Combes and Tate, 1976; Tate *et al*, 1978; Tate, McMurray, Riggs, Setaro and Horvath, 1980). The increase in mitochondrial variability of response to endurance training in the present study does suggest that the functional capacity of the mitochondria may be different in some manner from that noted with the sprint test.

Further study is necessary to clarify this point and the results would not only be important with regard to fatigue in exercise but certain parallels could be drawn with myopathies noted in clinical cases of idiopathic rhabdomyolysis (Scarpelli, Greider and Frajola, 1963; Savage, Forbes and Pearce, 1971; Lindholm *et al*, 1974).

METABOLISM STUDY

In exercising skeletal muscle the proportion of energy production derived from different extra and intra-muscular sources depends upon the intensity and duration of work (Gollnick, 1977). The intra-muscular substrates which are available for energy production are creatine phosphate, glycogen and triglyceride. In sprint exercise, the creatine phosphate stores are utilised at the onset of exercise after which energy is produced largely from anaerobic glycolysis, for which muscle glycogen is the principal substrate (Crabtree and Newsholme, 1972, 1975).

The regulatory pathways concerned with the degradation of carbohydrates are complex and involve nervous, hormonal and cellular stimuli. Studies mainly conducted *in vitro* (Review, Neely, Rovetto and Oram, 1972) have shown that carbohydrate utilisation is affected by intracellular factors stimulating or inhibiting glycolysis at the hexokinase, phosphorylase, phosphofructokinase and pyruvate dehydrogenase steps (Neely and Morgan,1974). Decreases in ATP and consequent increases in ADP, AMP and Pi, are shown to stimulate the PFK step (Passonneau and Lowry, 1962) whereas increased levels of ATP and CP on the other hand inhibit this step (Mansour, 1963; Krzanowski and Matschinsky, 1969). Changes in the ADP/ATP ratio are then considered to act as an important feedback signal regulating the rate of glycolysis and ATP formation. The PFK step can also be inhibited by an increase in fatty acid availability (Garland, Randle and Newsholme, 1963; Passonneau and Lowry,

1963) or by a lowering of pH (Danforth, 1965). Sprint exercise has been shown to decrease the muscle pH from 7.08 ± 0.04 at rest to 6.60 ± 0.14 at exhaustion (Sahlin, Harris, Nylind and Hultman, 1976). This decrease was linearly related to the muscle content of lactate and pyruvate.

As has already been noted the intensity and duration of work dictates the substrate utilisation (Gollnick, 1977). This section describes a study looking at this effect in horses which were being treated with anabolic steroids. The workloads selected were of a relatively high intensity and therefore the substrate measured was glycogen and the metabolites were lactate and pyruvate.

MATERIALS AND METHODS (2)

ANIMALS

The animals used in this study were horses numbered 1-6. They were undergoing training and anabolic steroid administration as outlined in Section 4.

FEEDING

Feeding was as outlined in Section 4 except that on the trial days the morning feed was delayed until after the animal had raced.

EXERCISE

All the trials were carried out during the anaerobic part of both cross-overs of the anabolic study. Each week the horses were galloped over 506m, 1025m and 1600m. These gallops were interspersed by a day of easy trotting and cantering for 20 minutes. Normally one of the sprint distances each week was run as an experimental trial. On the final week some of the horses were also tested over 3620m. Trials where biopsy samples were taken were, in chronological order,

a) 1025m (approx. 5F)

b) 1600m (approx. 8F)

c) 506m (approx. 3F)

d) 1025m (approx. 5F)

e) 3620m (approx. 18F).

The time lapse between each trial was approximately one week except in the d) to e) where the lapse was only 2 days. When the training session was an experimental trial, each animal ran individually and was ridden by the

same jockey.

MUSCLE SAMPLING

The percutaneous needle biopsy was used as already described to obtain the muscle samples. On all occasions the MG was the muscle sampled. The samples were taken on each occasion on the alternate side to the preceding trial. Previous work (Guy (1978) had shown that the right and left MG muscle did not differ in muscle composition. The area of skin for biopsy was prepared and two small scalpel incisions made, a minimal distance apart, while the horse was still in the box. The pre-exercise sample was taken just before the start of the gallop by which time the animal had trotted and cantered as a "warm-up". The post-exercise sample obtained was taken from the second incision immediately at the cessation of the gallop.

Resting samples were taken on a separate occasion with the horses in their boxes.

After biopsy the needle containing the sample was plunged directly into liquid N_2.

ANALYTICAL METHODS

The samples were analysed for glycogen, lactate and pyruvate by the methods outlined in Section 2.

TIMING

Times were taken over the varying distances for the majority of the animals.

STATISTICS

Comparison of the glycogen concentrations of anabolic and control groups was carried out using a paired

't' test except over the 3620m where sample numbers were especially small and comparison was made using a Student's 't' test. Similarly with the pyruvate and lactate samples paired data was not always available and therefore a Student's 't' test was used throughout.

RESULTS (2)

As the muscle samples were frozen while still in the biopsy needle, it was almost impossible to assess whether the sample was of sufficient quantity for analysis. It was necessary that both pre- and post-exercise samples should be obtained from any one animal and on many occasions only one sample was satisfactory. For this reason, the sample numbers over the various distances were smaller than would be expected.

GLYCOGEN

Resting glycogen levels vary throughout the training period and have been presented in Section 4. The variation in the pre-exercise samples, in this present section, was greater than that of the resting animal because of the variation in "warm-up" procedure and the possibility of an effect from a previous day's training. Varying decreases occurred in the total amount of glycogen utilised by each horse; however when these values were expressed as a percentage change from pre-exercise levels, the variation in results was reduced.

No significant differences existed between the anabolic or control group over any of the distances although the glycogen utilisation was slightly less in the anabolic group over each distance (Figure 5.9).

As there was no difference between control and anabolic group the figures have been combined to give a mean value for each horse. The mean of mean values was

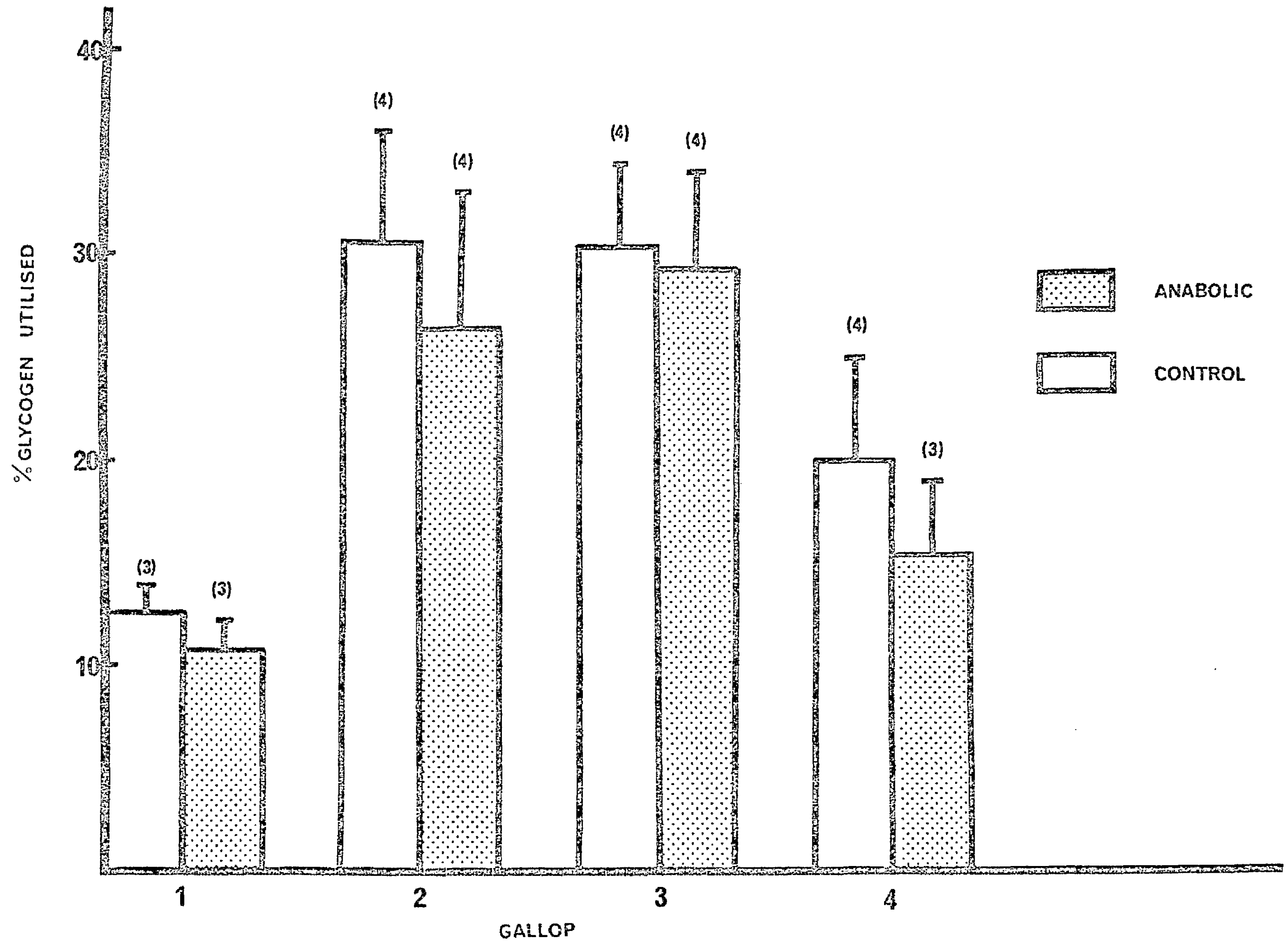
Figure 5.9 Percentage glycogen utilisation in the MG over various distances

Gallop 1 = 506m

Gallop 2 = 1025m

Gallop 3 = 1600m

Gallop 4 = 3620m


% GLYCOGEN UTILISED
40
30
20
10
(3)
(3)
(4)
(4)
(4)
(4)
(4)
(3)
ANABOLIC
CONTROL
1
2
3
4
GALLOP

then compared over the various distances (Table 5.3). The 506m trial utilised significantly less glycogen than the 1025m ($P < 0.05$) and the 1600m ($P < 0.02$). There were no other significant differences between the distances.

PYRUVATES

There was no significant difference between pre- and post-exercise pyruvate values over any distance. Nor was there any difference between controls and anabolic-treated groups. The resting mean value for the 6 animals was 0.45 ± 0.08 µmoles/g dry wt. tissue. A mean of mean value for the pre-exercise control group was 0.41 ± 0.04 µmoles/g dry wt. tissue. The post-exercise value for the same horses was 0.49 ± 0.03 µmoles/g dry wt. tissue. The anabolic group had a pre-exercise mean of mean value of 0.44 ± 0.06 µmoles/g dry wt. tissue and a post-exercise value of 0.44 ± 0.05 µmoles/g dry wt. tissue.

LACTATES

Resting mean value for the 6 horses was 8.3 ± 0.98 µmoles/g dry wt. tissue. No significant differences existed between the anabolic or control group over any of the distances, although 3 of the trials showed slightly less lactate production in the anabolic group (Figure 5.10). As there was no difference between the first and second 1025m trial, duplicate values for each horse have been averaged. No standard errors have been included in the 1600m trial in Figure 5.10 because of the small number and extremely large variation (control = 94.8 ± 42.8; anabolic 81.3 ± 50.8).

TABLE 5.3 Changes in glycogen and lactate concentrations in the MG over various distances (Mean ± S.E.M.)

Distance (m)	506	1025	1600	3620
% Glycogen utilisation	12.2 ± 0.6 (3)	28.7 ± 5.2 (4)*	29.7 ± 4.5 (4)**	20.4 ± 4.0 (5)
Lactate production µmoles/g dry wt. tissue	71.2 ± 7.8 (5) ††††	79.7 ± 9.3 (6) ††††	100.2 ±34.6 (2) †††	20.9 ± 2.2 (5)

* $P < 0.05$ when compared with the 506m trial.

** $P < 0.02$ when compared with the 506m trial.

††† $P < 0.01$ when compared with the 3620m trial.

†††† $P < 0.001$ when compared with the 3620m trial.

Figures in parenthesis are the numbers of samples.

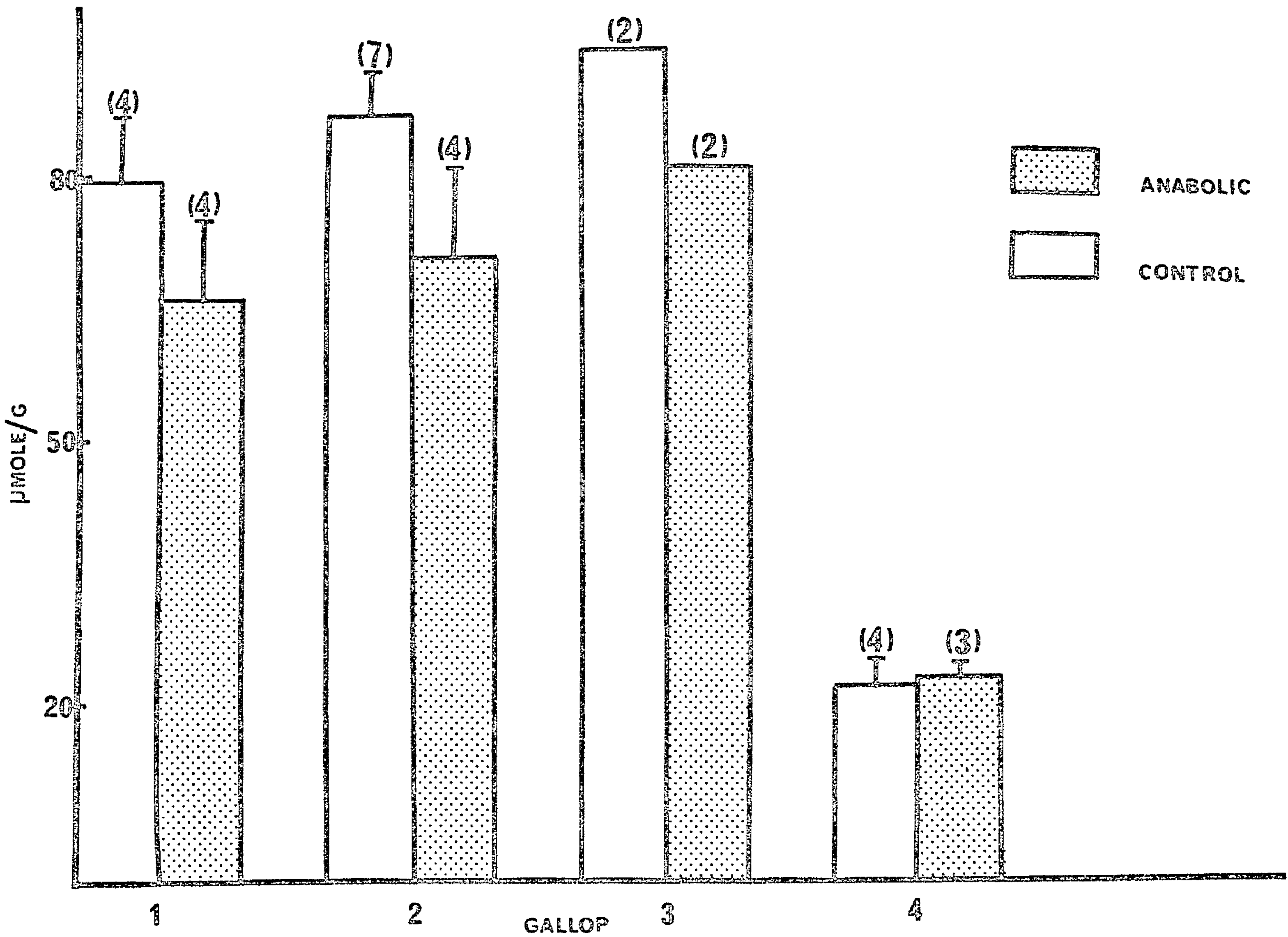
Figure 5.10 Lactate production (µmole/g) in the MG over various distances

Gallop 1 = 506m

Gallop 2 = 1025m

Gallop 3 = 1600m

Gallop 4 = 3620m



ANABOLIC
CONTROL
µMOLE/G
80
50
20
(4)
(4)
(7)
(4)
(2)
(2)
(4)
(3)
1
2
3
4
GALLOP

Mean values for each horse from the control and anabolic results have been combined in order to compare the lactate production over various distances (Table 5.3). The 3620m trial is significantly different from all other distances.

TIMES

Owing to practical difficulties some of the animals' performances were not timed. However, those for which there were results showed no consistent difference in performance between the control and anabolic group. The speed of the animals over the various distances and the glycogen utilisation per minute are given in Table 5.4. The numbers again are less than would be expected as those animals for which there were times did not always coincide with those for which there were glycogen values. The anabolic and control groups are combined and where there is a duplicate value for one horse a mean value has been taken. In addition, glycogen values, for which there were not paired (control and anabolic) data have been included.

TABLE 5.4 Glycogen utilisation (µmoles/g/min) over various distances

Distance (m)	n	Time (sec)	Speed ($M.sec^{-1}$)	Glycogen (µmoles/g)	Glycogen utilisation (µmoles/g/min)
506	3	34.9 ± 0.6	14.5 ± 0.2	86.9 ± 1.8	149.4 ± 0.8
1025	5	73.1 ± 1.7	14.1 ± 0.3	156.4 ± 23.0	129.3 ± 20.0
1600	4	127.2 ± 9.3	12.6 ± 0.4	137.9 ± 15.7	66.5 ± 8.0
3620	4	316.8 ± 6.9	11.4 ± 0.2	100.1 ± 14.7	18.8 ± 2.6

DISCUSSION

As has been found previously in the horse (Lindholm and Saltin, 1974; Guy, 1978) and other species (Bergström and Hultman, 1967; Reitman, Baldwin and Holloszy, 1973) exercise depletes the muscle of glycogen.

In this discussion the 506m trial is considered to be a maximum performance. Taking speed as an indication of the work intensity then the other distances are 97%, 89% and 79% of that maximum. As the intensity decreased so did the glycogen utilisation (μmoles/g/min) ($r = 0.70$, $n = 16$). Although the percentages represented here are not related to the maximum oxygen uptake (VO_2 max.) a similar correlation of glycogen utilisation with VO_2 max. has been found (Essén, 1977). Also at intensities greater than 90% of the VO_2 max. glycogen is the main fuel (Essén, 1977).

Direct comparison, in absolute terms, of the results in this study with other horse reports is difficult because of the wide variety of exercise régimes. It is also complicated by the fact that with repetitive trials over a distance (interval training) the initial trial has a higher glycogen utilisation than subsequent runs in both horse (Lindholm and Saltin, 1974; Guy, 1978) and human (Bergström and Hultman, 1967). Guy (1978) whose trial consisted of 4 x 500m gallops quotes a figure of 119.4 μmoles/g/min for an initial gallop over 500m compared to a mean value of 47.4 μmoles/g/min. for gallops 2-4. As

it has been noted that glycogen utilisation is related to pre-exercise levels then for comparison the results are best expressed in terms of utilisation as a percentage of resting levels. This gives values of 19% from the study of Lindholm and Saltin (1974), 34% from Guy (1978) and 28% in the present study. The percentages were calculated for the initial gallop in all cases. The reason for the lower value in the study by Lindholm and Saltin (1974) may be because the animals were Standardbred Trotters and the speed was slower. Also, different muscle groups are used by Trotters than by Thoroughbreds and it may be that the energy demand from the MG is not so great in trotting compared to galloping.

Comparison of the results with human reports is effectively more complex in that the large majority of maximal tests have been continued till exhaustion (Saltin and Karlsson, 1971; Karlsson, Diamant and Saltin, 1971). Studies on rats are similar (Baldwin, Campbell and Cooke, 1977). Approximating values from the paper of Saltin and Karlsson 88 µmoles/g/min. were utilised at 120% VO_2 max. Assuming that human muscle contains 300-400 µmoles glycogen/g (Essén, 1977) then the percentage glycogen utilisation would be in the range 22-29%. Using similar approximations for 90% VO_2 max. Karlsson *et al* (1971) report a figure of 27%. The percentage glycogen utilisation/minute, 28%, for the 506m trial and 29% for the 1025m trial are therefore in accordance with these observations.

Lactate and pyruvate resting values are comparable to those of man (Karlsson, 1970, 1971; Karlsson and Saltin

1970; Harris *et al*, 1974; Sahlin *et al*, 1976). The higher levels given in the study by Guy (1978) cannot be fully explained but may be due to different methodology. The lack of any change in muscle pyruvate levels with dynamic exercise is also in agreement with the literature (Sahlin *et al*, 1976) although some reports do give significant increases (Karlsson, 1971). Lactate levels in this study increased to approximately 80 μmoles/g dry wt. tissue in the 3 shorter distances. Assuming a conversion factor of 4 for the water content of skeletal muscle, Karlsson's (1971) results of lactate production after maximal exercise are approximately 70 μmoles/g dry wt. tissue. The higher lactate levels in the horse after exercise are in accordance with the results of higher blood lactate in horses than in man (Snow and Mackenzie, 1977). The reason for the equivalent lactate concentrations over 506m compared to 1025m in spite of lower glycogen utilisation is difficult to explain but may be related to the initial anaerobic period (oxygen deficit) associated with the onset of any exercise (Figure 1.1). During this period it is possible that a larger proportion of the glycogen breakdown is being converted to lactate until the increased muscular activity is matched by an increased metabolic rate and a new steady rate is reached.

The fuels used in any type of exercise are dependent upon the involvement of the different muscle types. Histochemical techniques have been employed in order to obtain information on substrate content and utilisation within

the different fibre types. Lindholm (1974) carried out such a study in the horse. Although this method of analysis is only semiquantitative, it gives valuable information about the qualitative picture in the muscle. It has been shown in the Standardbred horse (Lindholm, Bjerneld and Saltin, 1974) and in human (Essén, 1977) that all fibre types have a similar glycogen content.

Lindholm's results indicate that at slow trotting speeds the ST fibres were depleted of glycogen. When some ST fibres were depleted of glycogen the FTH fibres also became depleted. However, the studies were carried out at extremely low speeds (0.08 metres/sec.) and it was only after substantial depletion of the FTH and ST fibres that depletion of the FT fibres was noted. At increased speeds (12.5m/sec.) depletion in the FT fibres was as great as that seen in the FTH and ST. The physiological basis for this pattern of depletion is explained by the work of Kugelberg and Edström (1968)*. They found that motor neurones have different firing thresholds. Those recruited at low threshold are active more frequently, and for longer periods, than those recruited at high threshold It is generally believed that those with the lowest threshold are those associated with the ST and FTH fibres and this is therefore the reason that they are recruited at low intensity work. These fibres are high in mitochondrial content and consequently there is little lactate formation at slow speeds. However, at increasing work rates larger motor neurones are recruited and activate the FT fibres which have the lowest concentration of

* Henneman, Somjen and Carpenter, 1965 a,b, Henneman, Clamann Gillies and Skinner, 1974

mitochondria. The 506m and 1025m trial exemplify this theory in that large amounts of glycogen are utilised with subsequent anaerobic lactate production. The reason for the large variation in lactate production over the 1600m trial is most likely due to the fact that the horses can run this distance in two principal ways. One method is to start at near maximum intensity and then decrease speed rapidly towards the end. This would produce large quantities of lactate as the FT fibres would be recruited. Alternatively the animals could start at a lower intensity and maintain it throughout the race; low lactate levels would be produced as FT fibres would not be recruited to such a degree. In addition to varying fibre recruitment at different intensities it is also possible that other fuels may be used although at the high intensities used in this study, their role is probably minimal. These include the intramuscular triglycerides, blood glucose and free fatty acids in the blood. No measurement was made of the former substrate because of the large variation found within a muscle in the horse (Guy, 1978), but from human studies it is unlikely that these would be involved at workloads of such short duration (Essén, 1977).

Preliminary studies on the levels of blood glucose after the exercise bouts show no consistent differences between the various distances. Blood glycerol levels indicating the utilisation of FFA have not been carried out in this present study, although other studies have shown increases in glycerol concentrations after sprint

exercise in Thoroughbreds (Snow and Mackenzie, 1977).

It would appear therefore that the main substrate for energy in the 506m and the 1025m trial was glycogen. With the recruitment of the FT fibres at these high intensities this produced relatively high levels of lactate. The 3620m trial was of a lower intensity and thus the percentage recruitment of FT fibres may be less, the majority of activity being in the FTH and ST fibres. These fibres are well endowed with mitochondria and would therefore produce low lactate levels.

SECTION 6

GENERAL DISCUSSION

The price of a top class race horse may be over five hundred thousand pounds while stallions for stud may fetch up to ten million pounds. With this amount of money at stake it is obvious that every attempt is made to breed or train a winner. Yet, in spite of intensive efforts to do so, there has been little improvement in racing performance over the past 50 years. Few studies have looked closely at the Thoroughbred horse. The training methods and nutritional additives used at racing stables are largely anecdotal.

In 1977 Guy and Snow constructed a training programme for 4 Thoroughbred horses and looked at the effects on the skeletal musculature. This served as an excellent basis on which to expand the work to animals "in the field". In the past few years it has been possible to muscle biopsy a number of privately owned horses and to collate data relating fibre composition to the mode of work for which an animal has been selected (Snow and Guy, 1980).

One aspect which still needed investigation was the effect on the skeletal muscle of the widely abused anabolic steroids. Because, as in human studies, their use is banned in competing subjects in Great Britain, it was obvious that a "field" study was impossible. The most acceptable method of investigation was to examine the effects in a number of experimental animals. The cost of housing and feeding Thoroughbred horses is very expensive and therefore the number was limited, although by establishing

a cross-over protocol some of the problems associated with the small numbers were alleviated.

The study presented an insight into a variety of factors associated with training and training/anabolic steroid administration. However, the prime objective in administering anabolic steroids to healthy racing horses is to improve performance. The lack of any improvement in the anabolic-treated group compared to the exercising controls may be, because performance is dependent on such a variety of factors, that with small sample numbers any improvement would have been masked. Some effects of the anabolic steroids however were noted and it is possible to suggest how these could be used to improve an animal's performance.

The most consistent finding was the maintenance of the percentage FT fibres in the BF in conjunction with an increase in the aerobic capacity of the whole muscle. The Quarterhouse bred for its ability to cover short distances very quickly has a relatively high percentage of FT fibres whereas horses bred for longer distances have a high proportion of their fast twitch fibres as FTH (Snow and Guy, 1980). The animals treated with anabolic steroids in the present study would appear to have the qualities of both. Why the BF should be the only muscle to respond is difficult to explain but it could be due to a dependency on the residual androgenic activity of the steroid. Androgenic dependency is evident in muscles such as the levator ani and it may be that the skeletal muscles also show some specific

response to androgens. Further research on steroid receptors of skeletal muscles however would be necessary to clarify this point.

The idea that it is the residual androgenic activity of anabolic steroids which is the active component in producing an improved performance has been suggested elsewhere (Brooks, R.V., pers. comm.). However the difficulties in monitoring CNS effects have limited detailed studies in this area. In the present study, the behavioural changes, notably the increased aggressiveness in the geldings treated with anabolic steroids, could be channelled into an increased competitiveness. In a true racing situation, with other horses, this CNS-mediated effect could produce an improved performance.

Other aspects which may have aided performance in this study was the trend towards a lower glycogen utilisation and lower lactate production in the treated animals. Glycogen depletion has been noted to be one factor contributing to fatigue (Hultman and Saltin, 1967) and it is possible that the decreased glycogen utilisation would prolong the time taken to exhaustion in endurance events. The decreased lactate production with sprinting would partly prevent the decrease in muscle pH and the associated muscle dysfunction.

Alternative mechanisms by which anabolic steroids may aid performance have been suggested in other studies. It would appear that their effect is not associated with either changes in growth hormone, insulin (Galbraith, 1980), or glucocorticoids (Salmons, in press). One of the most

plausible mechanisms has been reported by Rogozkin (1979) and Rogozkin and Feldkoren (1979). These authors have shown that the activity of DNA-dependent RNA polymerase increases with anabolic steroid administration in rats. The enzyme provides for the primary synthesis of all RNA-types in skeletal muscle nuclei. The control animals in the study of Rogozkin and Feldkoren (1979) showed depression of this enzyme's activity after exercise. Anabolic steroids remove that depression. If this occurs in all species, then anabolic steroids may work to aid recovery after exhaustive exercise. This complies with observations of athletes themselves in that they have the ability to train more frequently, thereby producing an improved performance. In a study such as the present one, where control and treated groups are undergoing the same training programme, this would not be noted.

The possibility of using other steroids to promote muscle growth in animals has also been investigated. Castrate cattle have been shown to produce the greatest growth response with an oestrogenic steroid (Heitzman, 1976; Heitzman and Hartwood, 1977; Scott, 1978). In general, the cattle used have been immature and the value of an oestrogenic steroid to mature racing animals is speculative. The more obvious continuation from the use of anabolic steroids in sport is the use of testosterone and this is already evident in both human sport and horse-racing. The problems associated with administration of the natural male hormone are both complex and distressing notwithstanding the problems of controlling its use in sport.

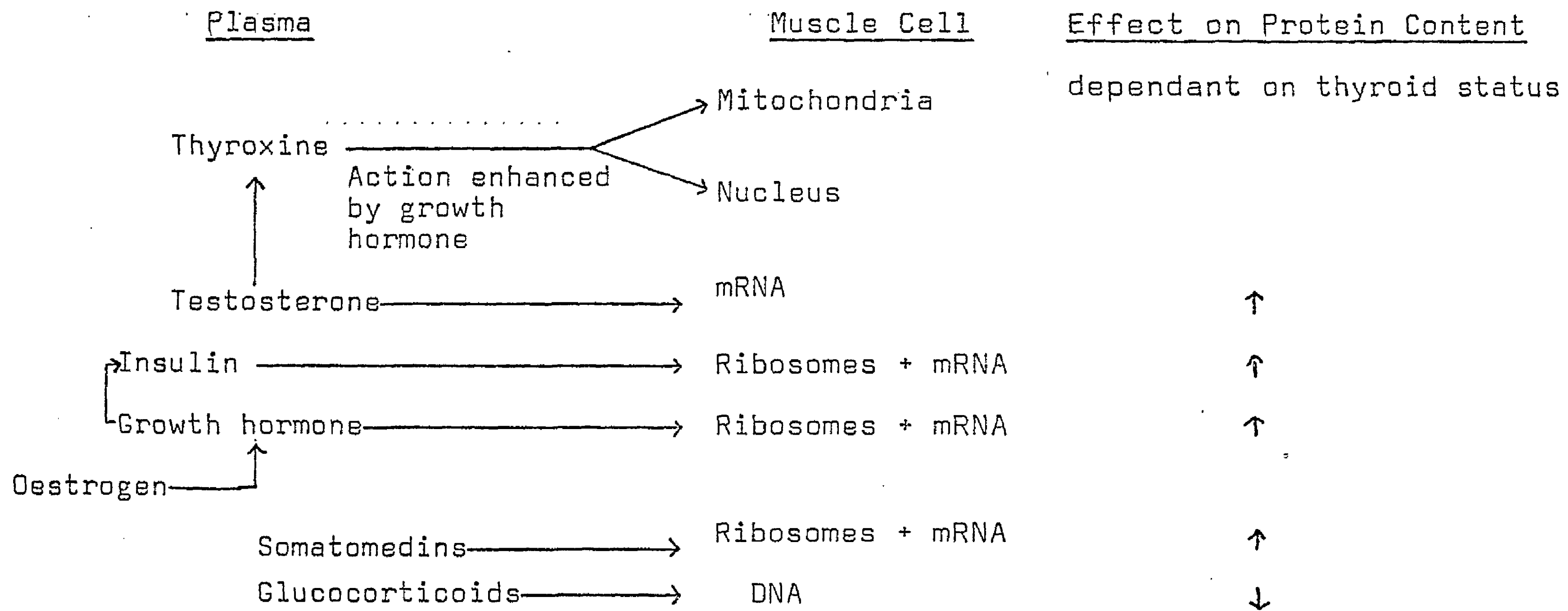
ADDENDUM

The Mechanism of Action of Androgenic Steroids.

It has been demonstrated by numerous workers that proteins in most organs are continually being synthesised and degraded and that the balance between the rate of synthesis and degradation is one of the major control factors of protein accretion. Hormones can influence muscle protein metabolism. Their effects may be summarised in Figure 1.

Anabolic steroids also cause an increase in protein content of skeletal muscle as measured by their nitrogen-retaining activity (Vander Waal, 1976) RNA content (Vernon and Buttery, 1978) and activity of RNA polymerase (Rogozkin and Feldkoren, 1979). However, whether the increased content results from altered rates of protein synthesis, degradation or both is confusing. Radiolabelled protein precursors have been used to study the rate of protein synthesis in various tissues, after castration and the administration of anabolic/androgenic steroids. Tissues that markedly decreased in weight after castration and increased after administration of anabolic/androgenic steroids exhibited a decrease and an increase respectively in the incorporation of the labelled precursors administered *in vivo* and also in cell free systems (Review Kochakian 1976). In intact female animals the administration of an anabolic steroid caused a decrease in protein synthesis and protein degradation (Buttery, Vernon and Pearson, 1978). However, the technique used to measure protein breakdown has been questioned by Millward *et al*, (1978). This group calculated protein breakdown by comparing accurate measurements of the rate of protein synthesis and

Figure 1



Action of Hormones on Muscle

Adapted from Buttery and Vernon (1980)

↑ increase

↓ decrease

of the rate of change of protein content, thus avoiding the traditional problems created by isotope reutilisation and by unwarranted assumptions about the kinetics of protein breakdown. Measuring breakdown in this way they found that protein breakdown increased in both catabolic and anabolic situations. The breakdown was faster in red oxidative muscles than in predominantly white muscles. Because the anabolic effect is dependent upon the muscle investigated (Kochakian and Tillotson, 1957), the sex, age and nutritional status of the animal and the dose rate administered (Heitzman, 1976) it is important that the measurement of the effects of anabolic steroids on protein synthesis and degradation should be carried out simultaneously. When these criteria are such that an anabolic steroid does cause an increase in protein content then it is of value to elucidate the mechanism of action.

The effect of anabolic hormones may be mediated via other hormones. In contrast to the oestrogenic growth promoters, the androgenic steroids are not associated with increases in either plasma levels of growth hormone or insulin (Galbraith, 1980) which would cause increased anabolism in the tissue. Subcutaneous implantation of androgens, in castrate male rats, cause suppression of leutinizing hormone releasing hormone (Cheung and Davidson, 1977). In addition, in steers, androgen implantation causes a decrease in plasma concentrations of tetraiodothyroxine (T_4) (Heitzman, 1976). This hypothyroid state may result in improved growth and feed utilisation (Reynolds, 1980) in addition to having direct effects on the skeletal musculature (Ianuzzo, Patel, Chen and

O'Brien, 1980; Johnson, Mastaglia, Montgomery, Pope and Weeds, 1980).

An alternative hypothesis to the effect of anabolic/androgenic steroids being mediated via other hormones is the possibility of anabolic/androgenic steroids acting directly on the skeletal muscle. The rat ventral prostate can be considered as the classical comparative model to study the direct action of androgens on a target organ. Testosterone is not the active form of the hormone although it can combine to chromatin directly *in vitro* (Mainwaring, 1979). In vivo it is converted to the more active 5 dihydrotestosterone by the cytoplasmic enzyme Δ^4-3-ketosteroid-5α -reductase (review Ofner, Leav and Cavazos, 1974). Cytosol binding studies have shown the affinity of 5αDHT to be ten times greater than the affinity of testosterone. In addition, the binding affinity of the receptor system is at least an order of magnitude higher than that of the plasma transport system. Testosterone therefore continues to enter the target cells and is metabolised until the intracellular receptor sites are saturated with locally formed 5α DHT. The receptor complex, in some way, becomes activated and in this form binds to nuclear chromatin. The receptor complex then triggers a series of processes fundamentally directed towards an increase in the transcription of the genetic information stored in DNA of the chromosomes. This ultimately results in protein synthesis (Mainwaring , 1979). In spite of many arguments that androgens could have a direct action on skeletal muscle

and so be responsible for the myotrophic effect, no evidence was presented until 1974 when Michel and Baulieu (1974) described androgenic/anabolic receptors in rat thigh muscle. In the absence almost totally of the enzyme Δ^4-3-ketosteroid-5α -reductase in skeletal muscle (Mainwaring, 1979) it is possible that testosterone might be active by itself.

Further evidence for the existence of androgen/anabolic receptors has both substantiated (Gustafsson and Pousette, 1975; Dubé, Lessage and Tremblay, 1976; Kreig and Voigt, 1977) and negated (Kreig, Szalay and Voigt, 1974; King and Mainwaring, 1974; Mayer and Rosen, 1975), Michel and Baulieu's (1974) original findings. However, King and Mainwaring (1974) point out that receptors may be present in skeletal muscle in quantities below the level of sensitivity of the available procedures. This point has been exemplified by Kreig and Voigt (1977) who by using radio-labelled 5α DHT and testosterone with a very high specific activity were able to demonstrate the existence of 5α DHT receptors in skeletal muscle where previously they had reported they did not exist (Kreig, Szalay and Voigt, 1974). However, even using such high specific activity ligands the results are questionable. It was not possible to characterise testosterone receptors and the Scatchard plot for 5α DHT from which the K_D and receptor numbers are calculated was estimated from 95% confidence limits of the regression line.

It is possible that the fraction isolated represents two types of receptor and if this was so it would be of pharmacological interest as receptor diversity may make it possible to distinguish myotrophic action from virilising activities and thus lead to the elucidation of an anabolic drug free from secondary effects. Supportive work, for the presence of androgen/anabolic receptors in skeletal muscle has been carried out with anabolic steroids in that 17β-hydroxestr-4-en-3-One-17β decanoate (retabolil) will compete with 19 nortestosterone for the cytoplasmic protein of skeletal muscle (Rogozkin and Feldkoren, 1979). Another direct mode of action may be by competing with glucocorticoids for the glucocorticoid receptors on skeletal muscle, thus reducing the catabolic effect of glucocorticoids (Mayer and Rosen, 1975). However experimental evidence now indicates that anabolic activity remains in adrenalectomised animals (Salmons, in press).

It is apparent therefore that this area of research warrants further attention before a comprehensible model for the action of androgenic/anabolic hormones on skeletal muscle can be elucidated.

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