BIOCHEMICAL ASPECTS OF EXERCISE IN THE HORSE

BY

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CONTENTS

		Page No.
GENERAL INTRODUCTION - LITERATURE SURVEY		1
SECTION I	- STUDIES ON THE KINETICS OF GOT, LDH, ALD AND CK PRESENT IN HORSE SERUM	
	Introduction	10
	Materials and Methods	13
	Results	20
	Discussion	58
SECTION II	- STUDIES ON THE EFFECTS OF EXERCISE ON SERUM ENZYME LEVELS IN THE HORSE	
	Introduction	65
	Materials and Methods	65
	Results	68
	Discussion	82
SECTION III	- STUDIES ON ISOENZYME PATTERNS OF HORSE SERUM AND TISSUES	
	Introduction	94
	Materials and Methods	96
	Results	105
	Discussion	123

Page No.

SECTION IV - EFFECT OF EXERCISE AND TRAINING ON BLOOD METABOLITES IN THE HORSE

Introduction 136

Materials and Methods 139

Results 147

Discussion 160

SECTION V - STUDIES ON THE BIOCHEMICAL EFFECTS OF CATECHOLAMINE ADMINISTRATION IN THE HORSE

Introduction 175

Materials and Methods 176

Results 180

Discussion 189

GENERAL DISCUSSION 199

REFERENCES 211

LITERATURE SURVEY

LITERATURE SURVEY

The training of racehorses varies markedly from trainer to trainer. In spite of the variability in the methods employed, most trainers are successful in that their horses do well in major racing events. There appears to be no universally recognised training schedule and, when it is recognised that it is just as important not to overtrain a horse and to have the animal in peak condition just at the time of the race, it would be desirable to have a scientific measure of the fitness of an animal.

Although several workers have investigated various physiological aspects of exercise in the horse (Matsuba and Shimamura, 1933; Radtsen, 1962; Bannister and Purvis, 1968; Littlewort and Hickman, 1968; Persson, 1968; Wittke and Bayer, 1968) relatively little work has been done on the biochemical aspects of exercise (Cardinet, Fowler and Tyler, 1963; Cornelius, Burnham and Hill, 1963; Carlson, Froberg and Persson, 1965; Cardinet, Littrell and Freedland, 1967) and each of these latter studies has been restricted to the measurement of one or two parameters. The present studies attempt to provide a more complete biochemical picture of the horse during exercise and training including effects on serum enzyme levels, serum isoenzyme patterns and blood metabolite levels.

The enzymes studied were chosen because each one is present in high concentration in horse skeletal muscle (Gerber, 1969). Since they are found mainly in the cell sap or cytoplasm, slight damage to muscle should result in their release into the circulation. The enzymes studied are (1) L-aspartate:α-oxo-glutarate aminotransferase (E.C.2.6.1.1) which will be referred to by its trivial name glutamic oxalacetic transaminase (GOT), (2) L-lactate: NAD^+ oxidoreductase (E.C.1.1.1.27) better known as lactic dehydrogenase (LDH), (3) ketose-1-phosphate aldehyde lyase (E.C.4.1.2.7) or aldolase (ALD) and (4) adenosine triphosphate:creatine phospho-transferase (E.C.2.7.3.2) or creatine kinase (CK). Some of these enzymes play an important role in the metabolism of muscle cells during exercise. Aldolase and lactic dehydrogenase participate in the oxidation of glucose to lactate with the simultaneous production of ATP. Although inefficient, this is the major mechanism for the production of energy in muscle under anaerobic conditions. Creatine kinase catalyses the formation of ATP from ADP and creatine phosphate stored within the cell, thereby ensuring a constant supply of ATP for mechanical work.

Under normal conditions cellular enzymes are found in serum at fairly constant low levels and it is generally believed that such enzymes are cell debris released into the bloodstream

by an unexplained mechanism and awaiting degradation and excretion (King, 1965). As such, these enzymes have no function in the circulation. The mechanism of removal from the bloodstream is also unclear since only minimal amounts of enzymes are excreted in the urine (Dunn, Martins and Reissmann, 1958) and bile (Hill, Nelson and Sowl, 1960). Evidence is accumulating to suggest that the reticulo-endothelial system may be involved (Fleisher and Wakim, 1963). Normal serum enzyme levels are maintained by a steady state of release from the cells and elimination from the serum. When this balance is upset by increased release from the cells, either as a result of cell necrosis or a change in cell membrane permeability, serum enzyme levels rise. Anything which results in cell damage, therefore, should produce elevated serum enzyme levels.

In man, elevated serum levels of GOT, LDH and ALD are found in a variety of conditions including myocardial infarction (LaDue, Wroblewski and Karmen, 1954; Volk, Losner, Aronson and Lew, 1956; Hamolsky and Kaplan, 1961), diseases involving the liver (Bruns and Puls, 1954; De Ritis, Coltorti and Giusti, 1955a, b; West, Heller and Zimmerman, 1958) and muscular dystrophy (Aronson and Volk, 1957; Schapira and Dreyfus, 1957). Muscular dystrophy and myocardial infarction also result in elevated CK levels (Dreyfus, Schapira and Demos, 1960; Dreyfus, Schapira,

Scebat, Resnais and Lenegre, 1960).

The use of serum enzymes as diagnostic tools in equine medicine has recently been reviewed by Gerber (1969) who found that GOT, LDH, ALD and CK become elevated in a variety of diseases but that all four enzymes increase dramatically in cases of equine paralytic myoglobinuria (Gerber, 1965a).

In addition to pathological conditions, serum levels of these four enzymes have been shown to increase as a result of strenuous exercise. Greatly increased serum levels of LDH and ALD have been observed following exercise in man (Fowler, Chowdhury, Pearson, Gardner and Bratton, 1962; Halonen and Konttinen, 1962) and in rats (Garbus, Highman and Altland, 1964). Griffiths (1966) has shown that serum creatine kinase activity in man is also dependent on the recent history of exercise. The effects of exercise on serum GOT in man are less clear, however, some workers reporting an increase (Schlang, 1961) and some a decrease (Critz and Merrick, 1962). The experiments on humans and rats also produced evidence that there is a significant difference in the serum enzyme increases after exercise in trained and untrained individuals.

Investigations in the horse have so far been restricted to monitoring serum levels of GOT during training (Cardinet et al, 1963; Cornelius et al, 1963) and to a limited study of CK and

GOT during exercise and training in one horse prior to an attack of equine paralytic myoglobinuria (Cardinet et al, 1967). The effects of exercise on serum GOT, LDH, ALD and CK concentrations in the horse were studied here to measure any changes in enzyme concentrations and to ascertain whether such changes can be related to the state of training of the animal.

From the literature, few workers appear to have attempted to determine the tissue of origin of any of the enzyme increased in serum as a result of exercise. Garbus et al (1964) observed the changes in the serum pattern of LDH iosenzymes in rats subjected to prolonged exercise. Like many other enzymes, lactic dehydrogenase is known to exist in more than one form. It is now known that animal tissues contain at least five different isoenzymes of LDH and that the relative concentration of these forms varies from tissue to tissue (Markert and Møller, 1959; Wieme, 1959; Wroblewski and Gregory, 1961). Since each tissue has a characteristic pattern of these isoenzymes and since the serum pattern reflects that of the damaged tissue, it should be possible to determine the site of such damage. Using this principle, Garbus et al (1964) concluded that, in rats, skeletal muscle is not the only tissue which contributes to the increase in serum LDH following exercise but that other tissues including heart and kidney are involved. To determine whether a similar situation

exists in the horse, serum LDH and CK isoenzyme patterns were examined before and after exercise and discussed in relation to tissue patterns. There appears to be a lack of information in the literature on the distribution of CK isoenzymes in the horse. However, tissue LDH isoenzyme patterns have previously been described by workers investigating the diagnostic significance of serum LDH isoenzymes in equine infectious anaemia (Coffman, Mussman and Cawley, 1969) and in equine paralytic myoglobinuria (Gerber, 1966). The different methods of isoenzyme separation and detection used by these workers have, however, led to different results.

In disease states the observed increase in serum enzyme levels is probably due to damage to the cell. In moderate physiological stress there is no visible damage to the cell and a change in cell membrane permeability has been postulated (Zierler, 1956; Cantone and Cerretelli, 1960; Highman and Altland, 1960). Over the last few years several workers have speculated on the nature of the stimulus which causes this change in the cell membrane. In the period 1956-58, Zierler demonstrated by *in vitro* studies that hypoxia, glucose lack and high potassium concentration can cause release of enzymes from tissue slices. The effect of hypoxia was subsequently confirmed by Highman and Altland (1960) by in *vivo* studies in dogs exposed to simulated high altitude.

Therefore, in parallel with the enzyme studies, the effects of exercise on some intermediary metabolites were also investigated. Much has been written about the metabolism of exercising muscle in the human and the effects of exercise on the concentration of glucose, pyruvate, lactate, ketone bodies and free fatty acids in the blood (Christensen and Hansen, 1939; Huckabee, 1958a & b; Carlson and Pernow, 1961; Astrand, Hallbäck, Hedman and Saltin, 1963; Havel, Naimark and Borchgrevink, 1963; Bergström and Hultman, 1967; Johnson, Walton, Krebs and Williamson, 1969). In comparison there is little information on this subject for the horse although Carlson et al (1965) have studied the role of lipid metabolism in horses subjected to mild exercise on a treadmill. The studies described were undertaken to determine the role of carbohydrate and lipid metabolism in aerobic and anaerobic conditions in the horse and to evaluate the contribution of tissue hypoxia to the release of intracellular enzymes into the circulation during exercise in this species.

Highman, Maling and Thompson (1959) have also shown that serum enzyme levels become elevated following administration of catecholamines to dogs. Although strenuous exercise is known to result in both tissue hypoxia (Huckabee, 1958b) and the release of catecholamines from the adrenal medulla and nerve endings (Gray and Beetham, 1957; Vendsalu, 1960) the effects of exercise on enzyme concentrations in the blood of the rat do not seem to be

attributable entirely to either of these stimuli (Garbus et al, 1964).

Since the mechanism of release of cellular enzymes into the bloodstream is, therefore, still unclear, the last part of this work was undertaken to investigate this aspect of the problem. The effects of catecholamine administration on serum enzymes, isoenzymes and blood metabolites were studied to determine the role of these hormones in the release of cellular enzymes in the horse. These studies would also provide data on the type of receptors mediating glycogenolysis and lipolysis in this species. According to Ahlquist (1948) adrenaline produces its effects by acting on two different types of receptor cells which have been termed alpha (α) and beta (β) cells. Despite recent evidence that the β group may, in fact, be subdivisible (Lands, Arnold, McAuliff, Luduena and Brown, 1967) this theory is still basically applicable. The types of receptors mediating glycogenolysis and lipolysis in man (Pilkington, Lowe, Robinson and Titterington, 1962; Antonis, Clark, Hodge, Molony and Pilkington, 1967), in the rat (Fleming and Kenny, 1964) and in the dog (Mayer, Moran and Fain, 1961; Kelly and Shanks, 1971) have been studied in detail and have been found to differ in some respects. Although muscle glycogenolysis and lipolysis are thought to be mediated by β-receptor cells in all three species, liver glycogenolysis appears

to be mediated by α-receptors in man and in the rat but by β-receptors in the dog. The use of various catecholamines would provide analogous information for the horse and this could indicate the relative importance of noradrenaline and adrenaline in mediating these responses during exercise in this species.

SECTION I

Studies on the Kinetics of GOT, LDH, ALD and CK present in Equine Serum

INTRODUCTION

The activity of an enzyme is influenced by many factors including substrate concentration, coenzyme concentration, pH and temperature. When the physical and chemical environment of the reaction mixture is optimal, the observed enzyme activity is a true measure of the enzyme concentration. It is widely accepted that enzymes derived from different species may vary somewhat in their optimal working conditions. Enzyme assay methods currently in use in routine laboratories, however, are based on the optimal working conditions for enzymes from the human and these conditions are not necessarily optimal for enzymes from other species. Since no information on some of the enzymes from the horse is available, the first section was devoted to the establishment of the optimal conditions for the assay of GOT, LDH, ALD and CK present in horse serum.

Of the methods currently available for the measurement of enzyme activity, those based on the recording of the initial rate of reaction under optimal conditions are to be preferred. Some methods in widespread use are based on measuring the change in some parameters, such as extinction, before and after a fixed time interval and, unless the reaction proceeds at a constant rate during the period of observation, the estimation of enzyme

activity may be invalid. Errors may arise in the assay of high activity sera where the reaction may be completed well within this period. In this instance the activity will be calculated on the assumption that the reaction is continuing throughout the observation period and the activity will be seriously underestimated. Non-linearity of an enzyme reaction can be a result of several factors including a decrease in substrate or coenzyme concentration, product inhibition, pH change or increase in the rate of the reverse reaction. Measurement of the initial reaction rate, when substrate concentration, pH etc., are relatively unchanged and the reaction is obeying zero-order kinetics, eliminates this error. For this reason, "initial rate of reaction methods" were used throughout this work.

In the past, considerable confusion has arisen over the definition and expression of enzyme activity. Different workers, often using the same assay procedure, have expressed activity in terms of milligrams, micrograms, millilitres, etc. of transformed substrate or as change in extinction or pH over different periods of time and at different temperatures. (The situation is further complicated by the fact that different laboratories may also use different assay methods making comparison of results difficult). To clarify this position, the "Commission on Enzymes of the International Union of Biochemistry" (1961) recommended the

adoption of the International Unit (I.U.) to define enzyme activity. The use of the International Unit, however, - defined as that amount of enzyme which transforms 1 micromole of substrate per minute under the defined conditions - may itself give rise to some confusion due to the term "under the defined conditions". Slight alterations to the conditions of assay of an enzyme may result in widely differing "International Units" although activities expressed in International Units are frequently compared without regard for the assay conditions. Despite these limitations, in order to comply with the Enzyme Commission's recommendation, one unit of activity was defined here as that amount of enzyme which transforms 1 micromole of substrate per minute under the conditions described. Serum enzyme activities, therefore, were expressed as milliunits per millilitre of serum, i.e. mI.U./ml.

In the present study, serum was used throughout in preference to plasma because of the reported inhibition of CK and perhaps LDH by certain anticoagulants (King, 1965). There is, in addition, no danger of serum clotting at any time during the assay procedures.

MATERIALS AND METHODS

Materials

The chemicals and enzymes listed below are those used in the experimental work described in this section. Unless otherwise stated, all reagents are "Analar" grade from BDH Chemicals Ltd., Poole, Dorset, U.K.

Substrates and coenzymes

Adenosine 5'-diphosphate (sodium salt) from Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Adeonsine 5'-monophosphoric acid.

L-aspartic acid.

Fructose-1,6-diphosphate (trisodium salt) from Boehringer Corp. (London) Ltd., London, W.5, U.K.

D(+) glucose.

Glutathione, reduced form (Sigma).

Iodoacetic acid.

Magnesium acetate.

β-nicotinamide-adenine dinucleotide, reduced form (NADH) disodium salt, from Boehringer Corp. (London) Ltd. The purity of each batch was checked using the molar extinction coefficient and was found to vary from 88-96 per cent.

β-nicotinamide-adenine dinucleotide phosphate ($NADP^+$) sodium salt (Sigma).

2-oxoglutaric acid.

Phosphocreatine, disodium salt hydrate (Sigma).

Sodium pyruvate (Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.).

Buffers

2:4:6 - collidine.

Potassium dihydrogen orthophosphate.

Triethanolamine hydrochloride (Sigma).

Enzymes

Glucose-6-phosphate dehydrogenase - lyophilised and sulphate free (Sigma). After reconstitution with distilled water, the activity was checked by the method described in the Boehringer Catalogue (1968). The reconstituted solution was stored frozen in small aliquots at -15°C.

Glycerol phosphate dehydrogenase in 2.0 M-ammonium sulphate from Boehringer Corp. (London) Ltd. The activity was checked using the method described in the Boehringer Catalogue (1968). The stock solution was found to be stable for several months when stored at 4°C.

Hexokinase - lyophilised and sulphate free (Sigma). The activity was checked by the method described in the Boehringer Catalogue

(1968). The activity of the reconstituted solution was found to decrease on storage at 4°C (the temperature recommended by the manufacturers). For this reason the solution was stored in small aliquots at -15°C.

Malate dehydrogenase in 3.2 M-ammonium sulphate from Boehringer Corp. (London) Ltd. The assay method described by Henry, Chiamori, Golub & Berkman (1960) was used to check the activity of the MDH solution. This solution was stored at 4°C for several months without loss of activity.

Triose phosphate isomerase in 2.8 M-ammonium sulphate from Boehringer Corp. (London) Ltd. The activity of the solution was checked by the method described in the Boehringer Catalogue (1968) and the stock solution stored at 4°C.

Methods

Collection and preparation of blood for enzyme analyses

Venous blood was obtained from the jugular vein. During this procedure the horse was restrained by means of a head collar and showed little signs of discomfort or excitement. When repeated sampling was required over a short period of time, the right and left jugular veins were used alternately. Catheterization of the vein was considered to be inadvisable because of the strenuous nature of the exercise to which the animal was subjected and the possibility of the catheter coming out during the exercise.

The blood was collected into polystyrene tubes and centrifuged at 2,500 r.p.m. (1,000 g.) for 5 minutes within 5 minutes of collection. When centrifugation was not possible, it was generally found that undisturbed blood separated naturally before clotting due to the rapid sedimentation rate of horse erythrocytes (Coffin, 1945). The upper plasma layer was decanted into glass bottles and allowed to clot. Using a glass rod with a rounded end, the clot was loosened from the sides of the bottle and left at room temperature to retract. After 1-2 hours, the serum was separated off into storage bottles and, unless enzyme analyses were carried out immediately, stored at -15^{o}C. If the sample clotted before the upper layer could be poured off, e.g. during centrifugation, careful "ringing" of only the upper layer reduced the risk of

haemolysis.

Before storage of the serum, it was essential to ensure that there were no erythrocytes present as these would rupture on freezing and thawing and possibly interfere with enzyme estimations. In the above procedure, the blood was separated prior to clotting but any contaminating erythrocytes in the serum were removed by centrifugation before freezing.

Serum for kinetic studies

Approximately 50 ml. of blood were taken from each of 6 normal horses and serum prepared as described. Each serum was stored frozen at -15°C in 2 ml. aliquots. In studying the kinetics of each enzyme, the effect of varying each individual parameter was determined using aliquots of 3 to 6 different sera as required.

Nicotinamide coenzyme-dependent assay methods

Each of the enzymes under study either utilizes a nicotinamide coenzyme in an oxidation/reduction reaction directly or 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 change in extinction per minute is readily converted into enzyme activity in milli International Units per millilitre of serum by the following equation:

$$\text{Enzyme activity (mI.U./ml)} = \frac{\frac{\Delta E}{\text{min}} \times V \times 1000}{\varepsilon \times v \times d} \quad \text{---} \ (1)$$

where E = change in extinction at 340 nm.

V = total reaction volume (in this case 3.0 ml)

ε = molar extinction coefficient of NADH/NADPH at 340 nm., i.e. 6.22 units/μ mole

v = volume of sample in millilitres

d = light path in centimetres.

All extinction measurements at 340 nm. were made using a double beam UV spectrophotometer (SP.800, Unicam, with a scale expansion unit and attached Servoscribe recorder). This allowed magnification of the change in extinction up to 20 times (although an expansion greater than X5 was never used because of background noise) thereby allowing more accurate measurement of small rates of change of extinction. The cells, 4 samples and 4 blanks (if necessary) were housed in a water-jacketed compartment fed from a water bath, the temperature of which was controlled to $\pm$ 0.1^{o}C. With this arrangement, the rates of reaction in 4 samples could be recorded simultaneously, since the instrument was programmed to record the extinction in each cell at 45 second intervals (Programme controller unit SP.825, Series 2, Unicam).

Enzyme Standards

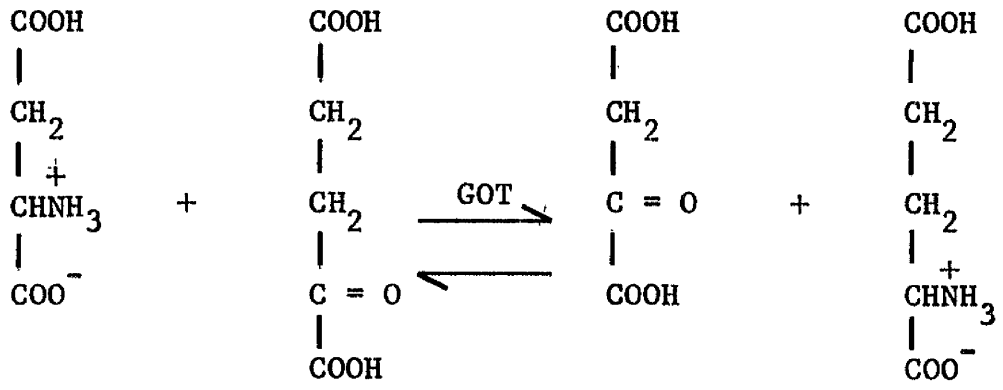
A standard is not required for the calculation of enzyme activity if the molar extinction coefficient of a

reaction substrate or product e.g. NADH, NADPH is known. It is advisable, however, to check the stability of the reagents daily by assaying a serum of known activity, in this case a pooled serum stored in small aliquots at -15°C.

RESULTS

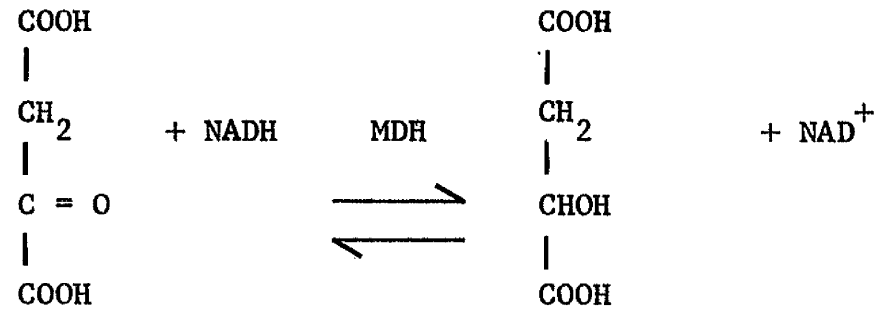
(1) Determination of Optimal Working Conditions of Horse Serum Glutamic-oxalacetic transaminase (GOT) - E.C.2.6.1.1

Principle of assay - GOT catalyses the following reaction:



Aspartate 2-oxoglutarate Oxalacetate Glutamate

The spectrophotometric method of assay is based on the conversion of one of the products, oxalacetate, to malate with the simultaneous oxidation of NADH to NAD^+:



Oxalacetate Malate

This is accomplished by the addition of excess NADH and malate dehydrogenase (MDH) to the assay mixture. Provided both NADH and MDH are present in excess, the rate of oxidation of NADH (with

subsequent decrease in extinction at 340 nm. measures the rate of production of oxalacetate i.e. GOT activity. The activity of this enzyme, therefore, is dependent on the concentrations of

(a) aspartate

(b) 2-oxoglutarate

(c) NADH

(d) MDH

and (e) buffer

and on the (f) pH

(g) temperature

and (h) enzyme concentration

of the reaction mixture. Each of these variables is dealt with individually below.

(a) Variation in rate with aspartate concentration

The reaction mixture contained, initially, 0.1M-phosphate buffer, pH 7.4, 6.7 mM 2-oxoglutarate, 0.12 mM-NADH (0.25 mg./3 ml. volume) and 40,000 units MDH, at a temperature of 32°C. The concentration of L-aspartate was varied from 6 mM to 334 mM and figure 1.1 shows the variation in rate of GOT reaction with aspartate concentration for a typical serum. Optimal rates were obtained from 150-334 mM concentration. In all subsequent assays a concentration of 167 mM-L-aspartate was used. The value of the Michaelis Constant (Km) was determined using the Lineweaver-Burk plot (1934). The Km for aspartate was estimated to be 1.02 x

EFFECT OF VARIATION IN ASSAY CONDITIONS ON THE REACTION RATE OF GOT IN HORSE SERUM

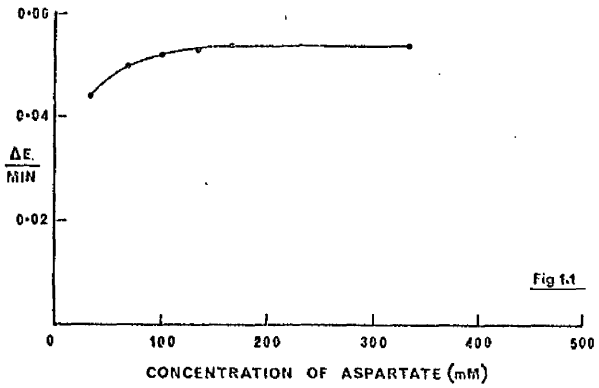


Fig 1.1

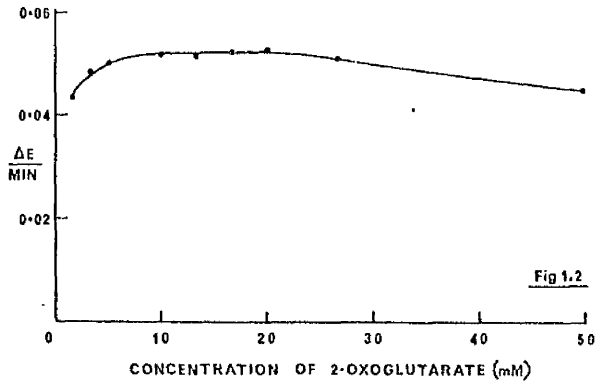


Fig 1.2

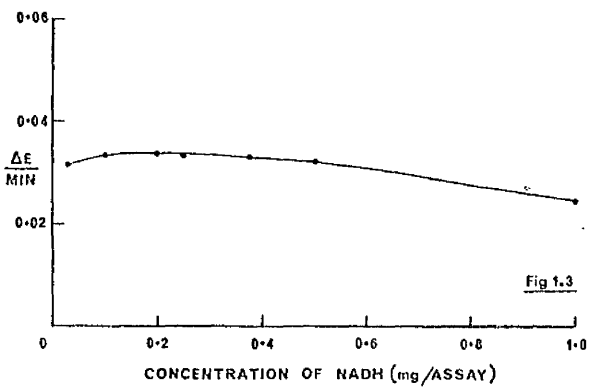


Fig 1.3

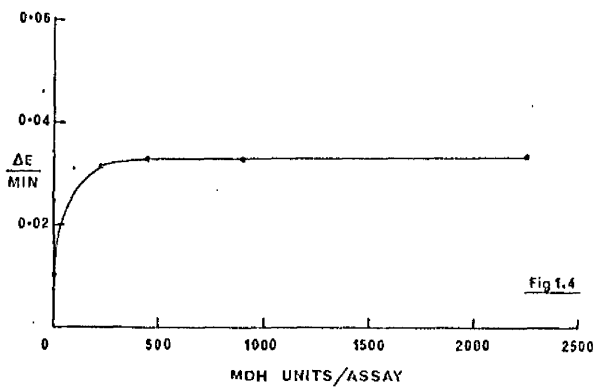


Fig 1.4

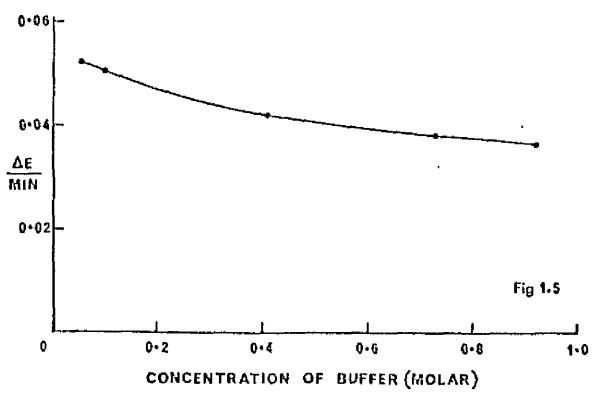


Fig 1.5

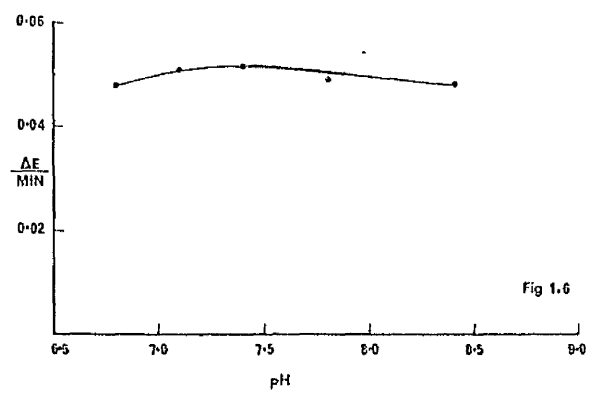


Fig 1.6

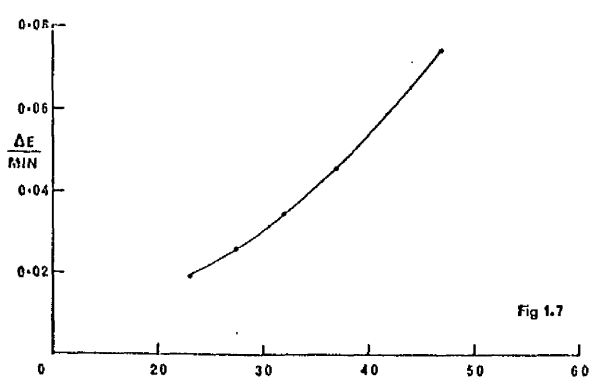


Fig 1.7

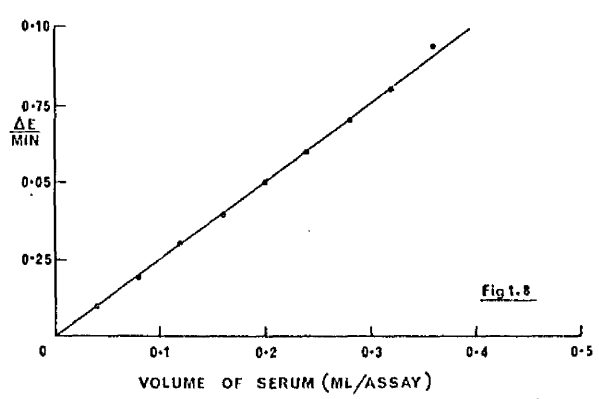


Fig 1.8

10^{-2}M ($\pm$ 0.15 x 10^{-2}M).

(b) Variation in rate with 2-oxoglutarate concentration

By varying the concentration of 2-oxoglutarate in the reaction mixture the optimal concentration was found to be 10 mM. Fig. 1.2 shows a typical curve for horse serum. For all the sera examined, inhibition occurred above 20 mM. Using the Lineweaver-Burk plot the Km for 2-oxoglutarate was calculated to be 3.8 x 10^{-4}M ($\pm$ 1.0 x 10^{-4}M).

(c) Variation in rate with NADH concentration

The change in rate of reaction with variation in NADH concentration is shown in Fig. 1.3 for a typical serum. Optimal rates were reached at a concentration of approximately 0.02 mg. NADH/assay (0.01 mM) for low activity sera (diluted) and 0.2 mg. NADH/assay (0.10 mM) for normal sera. In all subsequent assays a concentration of 0.25 mg/assay was used. Inhibition was observed at concentrations above 0.5 mg/assay (0.24 mM).

(d) Variation in rate with malate dehydrogenase (MDH) concentration

Known quantities of MDH were added to the reaction mixture and the corresponding rates measured (a typical curve is shown in Fig. 1.4). It can be seen that the MDH content of serum (no added MDH) was sufficient to allow considerable conversion of oxalacetate to malate although the reaction rates were still sub-optimal. The addition of 2,000 units of MDH to the assay mixture provided optimal rates, even for high activity sera (ΔE/min >/0.2).

(e) Variation in rate with buffer concentration

The effect of varying the concentration of phosphate buffer while maintaining the pH at 7.4 is illustrated for a typical serum in Fig. 1.5. For the sera examined, the activity decreased with increasing phosphate concentration. In all subsequent assays a 0.1M-phosphate buffer was used (the final phosphate concentration for a 0.2 ml. sample volume actually being 0.093M).

(f) Variation in rate with pH

Fig. 1.6 illustrates the variation in activity with pH for a typical serum at a constant phosphate concentration of 0.1M. The activity increased to a maximum in the range 7.3-7.5 for each serum examined.

(g) Variation in rate with temperature

The effect of varying the temperature from 23°C to 47°C was examined. Fig. 1.7 shows the result for a typical serum. As expected, the activity of each serum increased with temperature and the temperature finally chosen for all subsequent assays was 37°C.

By carrying out the assay at 37°C, however, the possibility of some thermal denaturation of the enzyme during the incubation period was increased. To investigate this, the incubation period was varied (using the same serum) from 5 minutes to 45 minutes and the enzyme activity measured as usual. The

stability of GOT was found to be such that, even after an incubation period of 45 minutes at 37°C, there was no detectable loss of activity in the sample.

To allow conversion of activity at other commonly used temperatures, e.g. 25° and 32°C to that at 37°C, temperature conversion factors were calculated. Arrhenius plots of log ΔE/min against absolute temperature were made for 4 sera and the slopes calculated. These ranged from -2460 to -2560 with a mean of -2515. The factors (f) to convert activity at temperature X to 37°C were calculated from the slope of the Arrhenius plot by the equation:-

$$\log f = \text{slope} \times 10^{-3} \times \left[\frac{1000}{Tx} - \frac{1000}{310.18} \right]$$

where Tx = absolute temperature in°K.

The temperature conversion factors from 20°C to 40°C are shown in Table I.I.

(h) Variation in rate with enzyme concentration

Reaction rates were proportional to enzyme concentration as determined by varying the amount of serum used in the test from 0.04 ml. to 0.40 ml. (ΔE/min ranging from 0.010 to 0.208) - Fig. 1.8. For the typical serum shown, the straight line passes through zero indicating that any "blank" activity is negligible.

TABLE I.I FACTORS FOR CORRECTION TO 37°C

Temperature of Reaction °C	Factors (f)			
	GOT	LDH	ALD	CK
20	2.96	3.05	4.54	4.98
21	2.76	2.84	4.14	4.51
22	2.58	2.66	3.77	4.08
23	2.42	2.48	3.44	3.70
24	2.26	2.32	3.13	3.36
25	2.12	2.17	2.86	3.05
26	1.99	2.03	2.61	2.77
27	1.86	1.90	2.39	2.52
28	1.75	1.78	2.18	2.29
29	1.64	1.66	2.00	2.08
30	1.54	1.56	1.83	1.89
31	1.45	1.46	1.67	1.73
32	1.36	1.37	1.53	1.57
33	1.28	1.29	1.41	1.44
34	1.20	1.21	1.29	1.31
35	1.13	1.13	1.18	1.20
36	1.06	1.06	1.09	1.09
37	1.00	1.00	1.00	1.00
38	0.94	0.94	0.92	0.91
39	0.89	0.88	0.85	0.84
40	0.84	0.83	0.78	0.77

Optimal Conditions for the Assay of Horse Serum GOT

As a result of the above studies, the final conditions of assay chosen were: 167 mM-L-aspartate, 10 mM-2-oxoglutarate, 0.12 mM-NADH and 2,000 units MDH in 0.1M-phosphate buffer, pH 7.4 at 37°C. The final volume was 3.0 ml.

Reagents

1M-phosphate buffer, pH 7.4:- 136 g. KH_2PO_4 were dissolved in approximately 800 ml. distilled water, adjusted to pH 7.4 with 4M-NaOH and made up to 1 litre.

0.1M-phosphate buffer, pH 7.4:- Diluted from 1M buffer with distilled water and the pH checked.

0.5M-L-aspartate in 0.1M buffer, pH 7.4:- 13.3 g. L-aspartate were dissolved in approximately 80 ml. water and 80 ml. 1M-NaOH by warming in a steam bath. After cooling to room temperature, 20 ml. 1M-phosphate buffer were added. The pH was adjusted to 7.4 with 1M-NaOH and the volume made up to 200 ml. with water. (Stored frozen at -15°C).

0.3M-2-oxoglutarate in 0.1M buffer, pH 7.4:- 2.19 g. 2-oxoglutarate were dissolved in approximately 30 ml. water and 5 ml. 1M-phosphate buffer, pH 7.4. The pH was adjusted to 7.4 with 1M-NaOH and made up to 50 ml. (Stored frozen at -15°C).

3.6mM-NADH in 0.1M buffer, pH 7.4:- 2.5 mg/ml. NADH (corrected for purity) were dissolved in 0.1M-phosphate buffer, pH 7.4. (Made up fresh daily).

MDH solution, 20,000 units/ml:- Stock solution was diluted with 0.1M-phosphate buffer to yield 20,000 units/ml. Dilute solution was prepared daily.

Assay procedure

1.5 ml. 0.1M-phosphate buffer, 1.0 ml. aspartate solution, 0.1 ml. NADH solution, 0.1 ml. MDH solution and 0.2 ml. serum were added to a quartz cuvette. The solution was mixed and incubated for 15 minutes at 37°C in the spectrophotometer. When the extinction was steady, the reaction was started by adding 0.1 ml. 2-oxoglutarate (warmed to 37°C in the water bath). After mixing, extinction readings were recorded for 5 minutes and the enzyme activity calculated from the ΔE/min. For ΔE/min. above 0.20 the sample was diluted with 0.1M-phosphate buffer and the assay repeated.

During the incubation period, the extinction of the assay mixture decreased as a result of the oxidation of NADH by serum (and added) dehydrogenases using endogenous keto acids as substrates e.g. in the above assay mixture, endogenous oxalacetate would be reduced by the added MDH. After 15 minutes these side reactions had ceased and the extinction was steady. There was, in fact, a very slow decrease in extinction even after 15 minutes incubation, due partly to the spontaneous degradation of NADH and partly to the continued oxidation of NADH at a very slow rate by side reactions. Since this residual extinction decrease amounted to less than 1% of the

GOT activity of the samples, it was ignored. Using this procedure, therefore, no blanks were necessary.

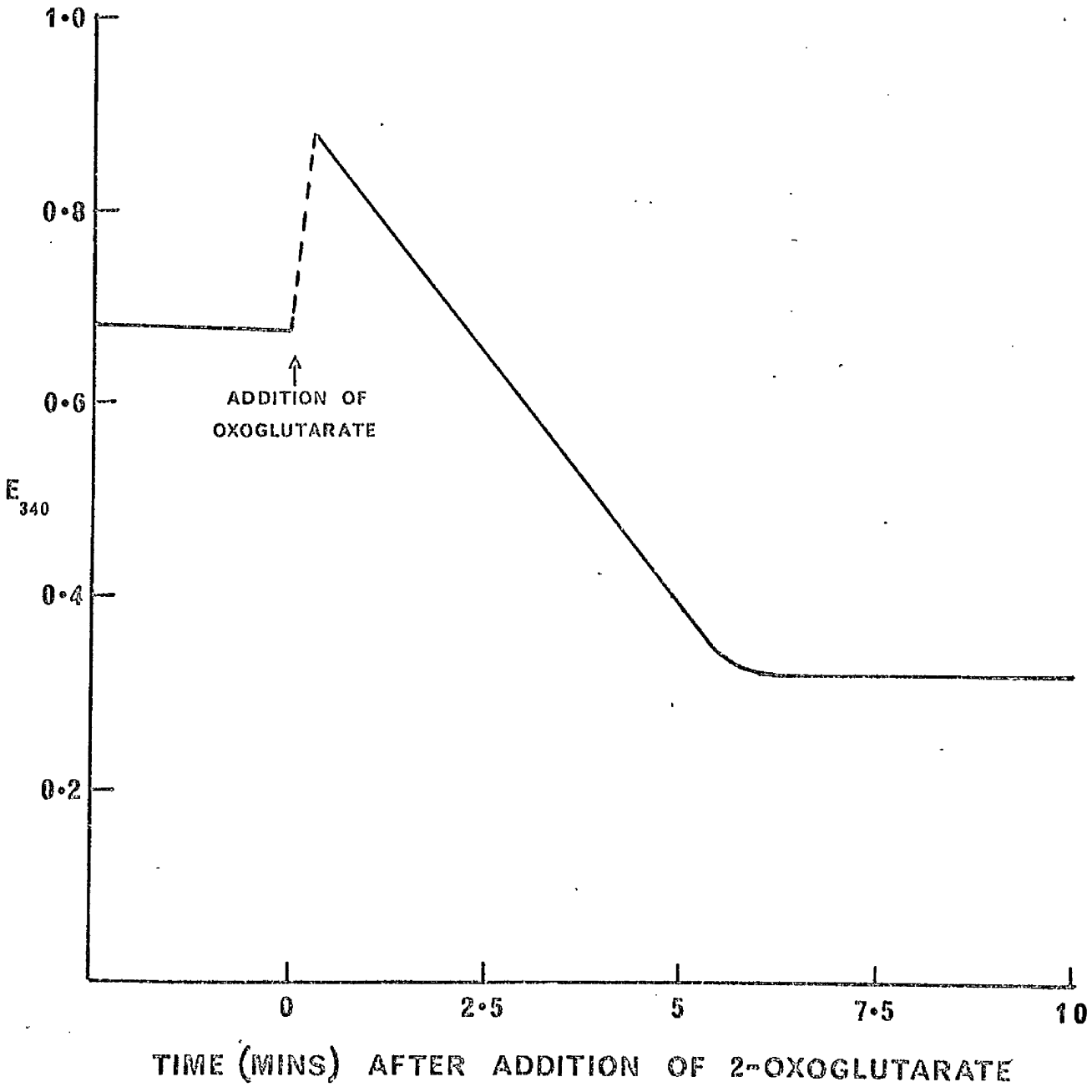
Linearity of reaction

Using the above procedure, the reaction was linear for a period of 5 to 10 minutes depending on the activity of the serum. A diagnostic representation of the progress of the GOT reaction is shown in Fig. 1.9. The "lag phase" of several minutes described by Henry et al (1960) was not observed. According to Henry, this "lag phase" is the time required for the accumulation of oxal-acetate to a stage where it is removed by the MDH reaction as quickly as it is formed i.e. a steady state exists. This being the case, the GOT activity of a normal horse serum would be high enough to reduce the lag period to a few seconds - a time less than that between addition of 2-oxoglutarate and recording of the extinction. Under these circumstances, no "lag phase" would be seen.

Reproducibility of method

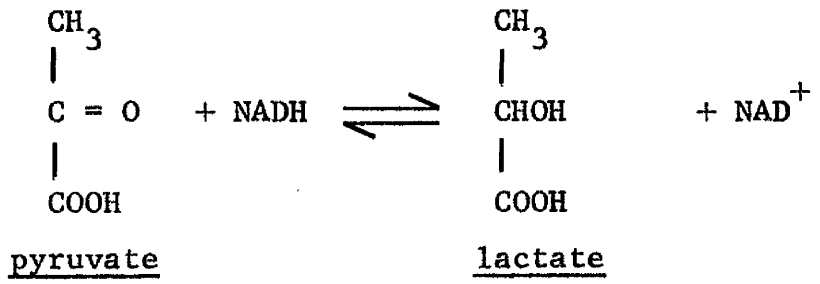
Of 22 samples assayed in duplicate the mean difference between duplicates was calculated to be 4.3 mI.U/ml.The mean of 8 measurements on the same serum sample was 259 $\pm$ 4.8 mI.U./ml. with a coefficient of variation of 1.9%. Daily assays performed on frozen aliquots of the same serum for 7 days gave a mean of 254 $\pm$ 9.3 mI.U/ml. with a coefficient of variation of 3.7%.

Fig 1.9 REACTION PROGRESS CURVE FOR GOT



(2) Determination of Optimal Working Conditions of Horse Serum Lactic Dehydrogenase (LDH - E.C.1.1.1.27)

Principle of assay - LDH catalyses the reversible reduction of pyruvate to lactate with the concomitant oxidation of NADH to NAD^+:



pyruvate lactate

The enzyme may, therefore, be assayed in the direction of pyruvate to lactate or lactate to pyruvate. In this work, the former direction was chosen, i.e. from pyruvate to lactate. The rate of decrease of extinction at 340 nm. is a measure of LDH activity. The influence of the concentration of

(a) phosphate buffer

(b) pyruvate

(c) NADH and of

(d) pH

(e) temperature and

(f) enzyme concentration

on the enzyme activity is discussed below.

(a) Variation in rate with phosphate buffer concentration

The initial conditions used were - 0.5 mM-pyruvate, 0.5 mg.

NADH/assay (0.25 mM) in phosphate buffer, pH 7.4 in a 3 ml. volume at 37°C. The concentration of phosphate in the assay mixture was varied from 10 mM to 473 mM while maintaining the pH at 7.4 and the effect is shown for a typical serum in Fig. 1.10. Horse serum LDH showed maximal activity at phosphate concentrations around 0.1-0.3M.

In all subsequent assays, a 0.1M-phosphate buffer was used.

(b) Variation in rate with pyruvate concentration

Fig. 1.11 illustrates the effect of increasing the pyruvate concentration from 0.1 mM to 1.0 mM. Optimal activity was obtained at pyruvate concentrations around 0.8-1.0 mM and in all subsequent assays, a concentration of 1.0 mM was used.

The Michaelis constant-Km (pyruvate) for equine LDH was calculated, using a Lineweaver-Burk plot (1934), to be $1.4 \times 10^{-4} \pm 0.33 \times 10^{-4}$M.

(c) Variation in rate with NADH concentration

The change in rate of reaction with increasing concentration of NADH is shown for a typical serum in Fig. 1.12. Although optimal activity was provided by the addition of 0.3 mg. NADH/assay (0.14 mM), the concentration chosen for use in subsequent assays was 0.4 mg./assay (0.19 mM). This concentration supported a linear reaction for at least 10 minutes even with high activity sera (ΔE/min. > 0.100).

EFFECT OF VARIATION IN ASSAY CONDITIONS ON THE REACTION RATE OF LDH IN HORSE SERUM

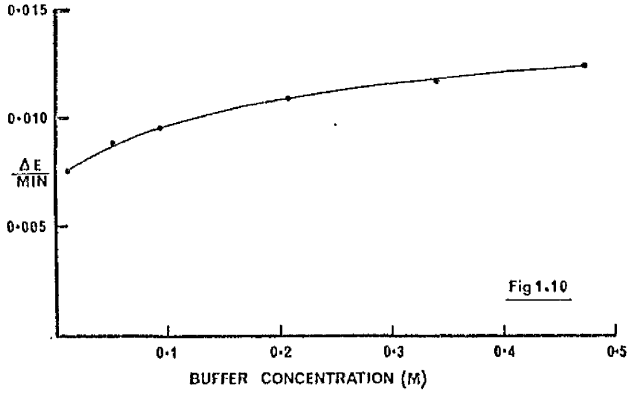


Fig 1.10

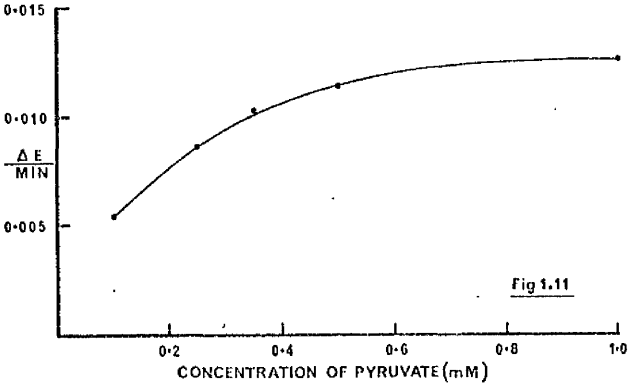


Fig 1.11

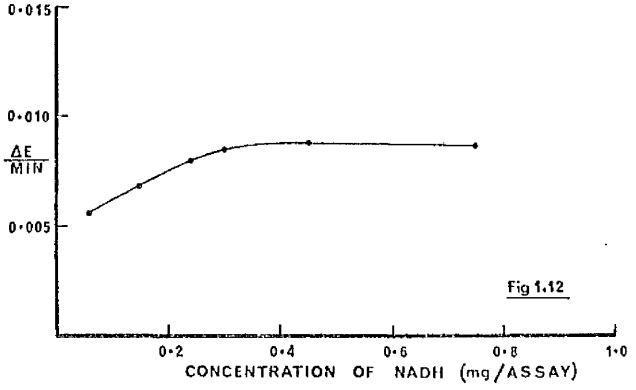


Fig 1.12

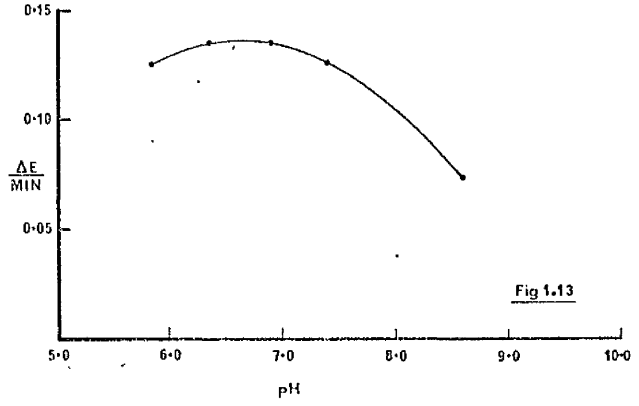


Fig 1.13

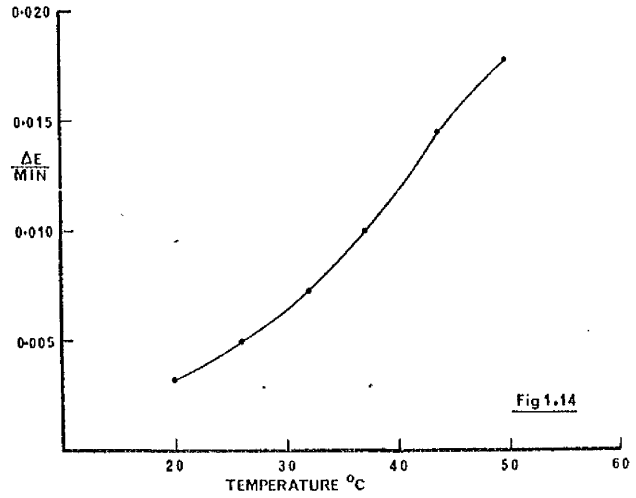


Fig 1.14

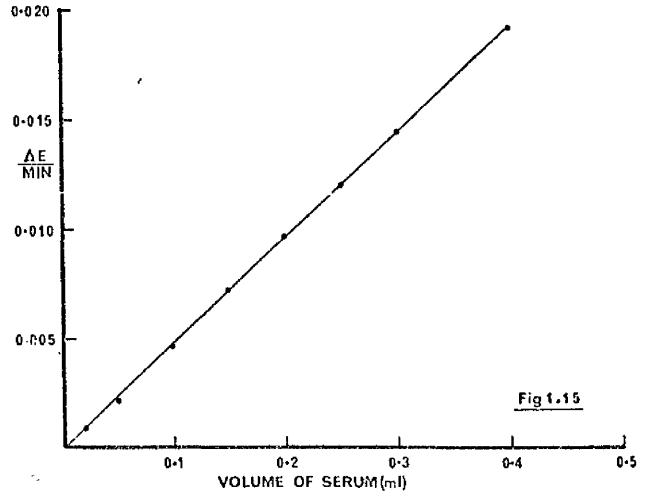


Fig 1.15

The Km (NADH) of equine LDH was estimated to be 2.4 x $10^{-5} \pm 2.13 \times 10^{-5}$M.

(d) Variation in rate with pH

Fig. 1.13 illustrates the variation in activity with pH for one serum. The optimal pH was taken as 7.0 for all subsequent assays although the optimum seems to vary with different sera.

(e) Variation in rate with temperature

Enzyme activity was shown to increase with increasing temperature in the range 20°C to 49.5°C. Fig. 1.14 shows a typical rate against temperature graph for LDH. The temperature for all subsequent assays was 37°C (at this temperature there was no detectable loss of activity during incubation even after an extended incubation period of 45 minutes). An Arrhenius plot gave a mean slope of -2588 ± 98, and using this information, factors to convert LDH activity at other temperatures to that at 37°C were calculated and are shown in Table I.I.

(f) Variation in rate with enzyme concentration

The concentration of enzyme was varied by increasing the volume of serum (diluted 1:10 or 1:20 with 0.1M-phosphate buffer, pH 7.0) from 0.02 ml. to 0.40 ml. For the sera examined (Fig.1.15 shows the results for one) reaction rates were proportional to enzyme concentration. For most normal horse sera 0.02 ml. serum (0.20 ml. of 1:10 dilution) was a convenient volume for LDH assays.

Optimal Conditions for the Assay of Horse Serum LDH

As a result of the above studies, the final conditions chosen for the assay of LDH in horse serum were:- 1 mM-pyruvate, 0.19 mM-NADH in 0.1 M-phosphate buffer, pH 7.0 at 37°C. The final volume was 3.0 ml.

Reagents

1.0 M-phosphate buffer, pH 7.0:- 136 g. KH_2PO_4 were dissolved in approximately 600 ml. distilled water and adjusted to pH 7.0 with 4 M-NaOH. The solution was made up to 1000 ml. with distilled water and the pH checked. (Stored at 4°C).

0.1 M-phosphate buffer, pH 7.0:- Diluted from 1.0 M buffer with distilled water and the pH checked. (Stored at 4°C).

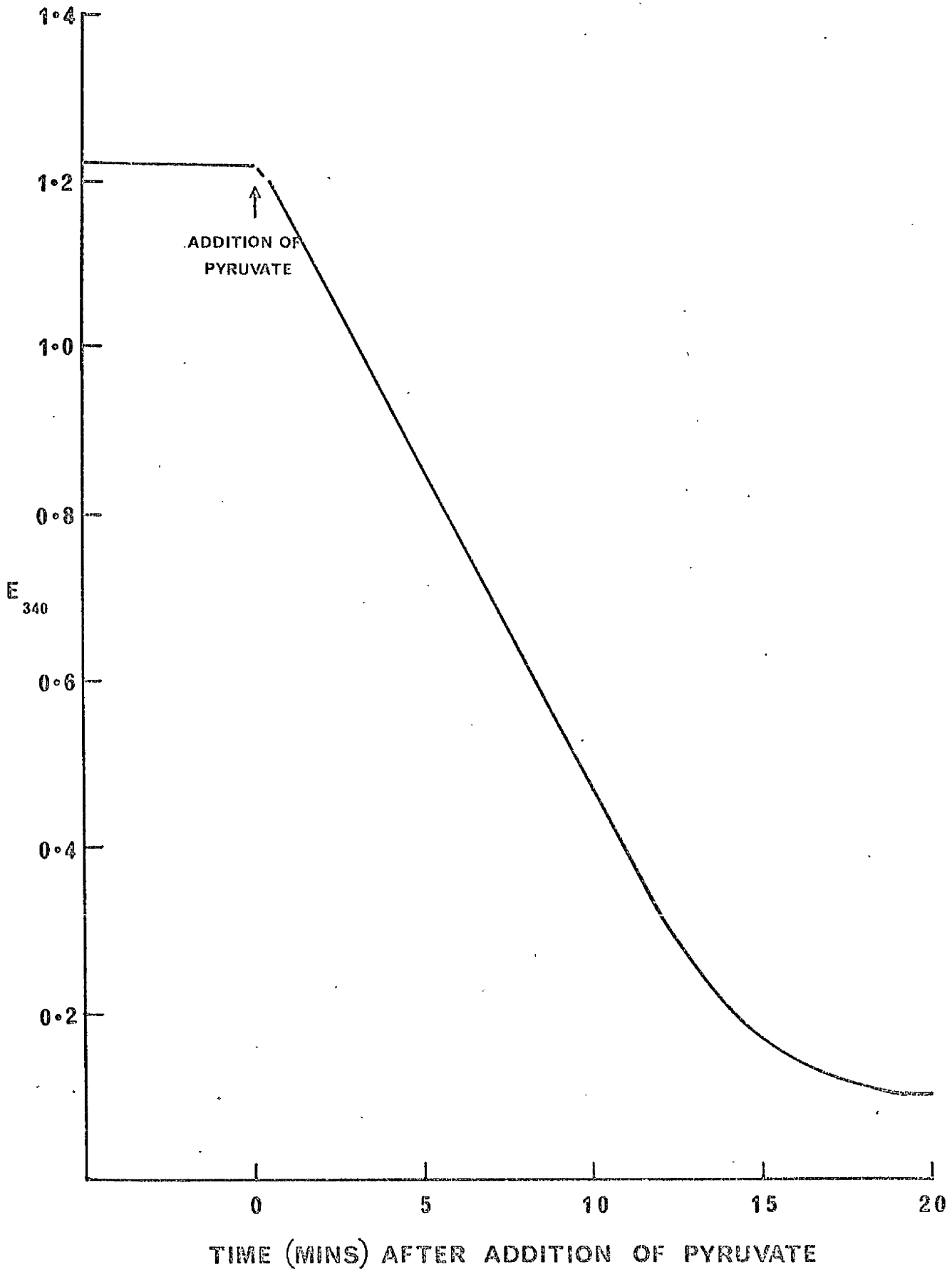
0.03 M-pyruvate in 0.1 M buffer:- 66 mg. sodium pyruvate (Koch-Light Laboratories) were dissolved in 20 ml. 0.1 M-phosphate buffer. (Stored frozen at -15°C).

5.6 mM-NADH in 0.1 M buffer:- Sufficient NADH-disodium salt (Boehringer) to give a concentration of 4.0 mg/ml. was dissolved in 0.1 M buffer. (Made up fresh daily).

Assay Procedure

2.6 ml. 0.1 M-phosphate buffer, 0.1 ml. NADH solution and 0.2 ml. sample (a 1:10 dilution of serum with 0.1 M buffer) were added to a quartz cuvette and mixed. After incubating at 37°C in the spectrophotometer for 15 minutes or until the extinction had

Fig 1.16 REACTION PROGRESS CURVE FOR LDH



stopped decreasing, the reaction was started by the addition of 0.1 ml. pyruvate solution (pre-warmed to 37°C). The solution was mixed and extinction readings recorded for 5 minutes. The LDH activity of the serum was calculated from the ΔE/min.

Using this procedure, the reaction was linear for at least 10 minutes after addition of pyruvate. A diagrammatic representation of the reaction progress curve for LDH is shown in Fig. 1.16.

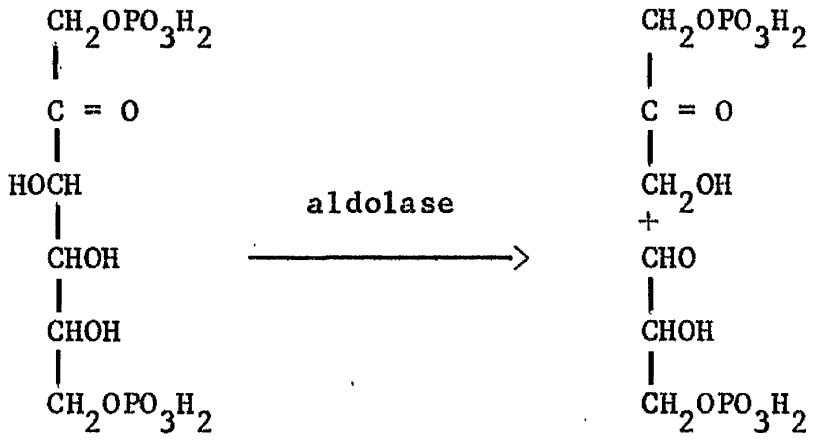
Reproducibility of Method

When 50 assays were performed in duplicate, the mean of the differences between duplicates was calculated to be 14.3 mI.U./ml.

The mean of 9 replicate measurements made on one serum was 884 $\pm$ 20.5 mI.U./ml. with a coefficient of variation of 2.3%. The variation from day to day was estimated by assaying frozen aliquots of the same serum on 7 days to give a mean of 717 $\pm$ 30 mI.U./ml. (coefficient of variation 4.2%).

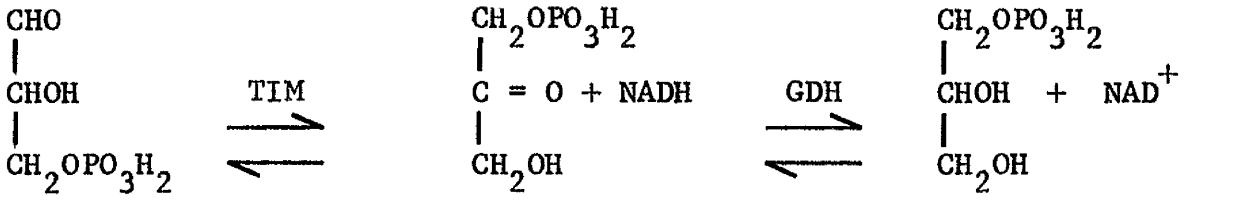
(3) Determination of Optimal Working Conditions of Horse Serum Aldolase (ALD) - E.C.4.1.2.7

Principle of assay - Aldolase catalyses the cleavage of fructose - 1,6-diphosphate into two triose phosphates:-



fructose-1,6-diphosphate

The reaction is monitored by converting glyceraldehyde-3-phosphate produced in this reaction to dihydroxyacetone phosphate by the action of triose phosphate isomerase (TIM). This is, in turn, reduced by glycerol phosphate dehydrogenase (GDH) to glycerol phosphate with the simultaneous oxidation of NADH e.g.:



glyceraldehyde-3-phosphate | dihydroxyacetone phosphate | glycerol phosphate

Provided the auxiliary enzymes and NADH are present in excess, the rate of oxidation of NADH is proportional to the aldolase activity.

The rate of the aldolase reaction is influenced by the concentration of:

(a) buffer

(b) fructose diphosphate

(c) iodoacetate

(d) NADH

(e) GDH

(f) TIM and by

(g) pH

(h) temperature and

(i) enzyme concentration as described below.

(a) Variation in rate with buffer concentration

The effect of varying the concentration of phosphate and collidine buffers was examined. The initial conditions were 5 mM-fructose-1, 6-diphosphate, 0.3 mM-iodoacetate, 0.25 mg. NADH/assay (0.12 mM), 0.8 units GDH/assay and 48 units TIM/assay in buffer at pH 7.4. Although the enzyme activity remained reasonably constant with increasing concentrations of phosphate buffer (Fig. 1.17 shows the results for one serum), the collidine buffer system produced a pronounced inhibitory effect on the reaction rate. As a result a 0.1 M-phosphate buffer was used in all subsequent assays.

(b) Variation in rate with fructose-1, 6-diphosphate concentration

The concentration of fructose-1, 6-diphosphate in the assay mixture was varied from 0.2 mM to 10 mM and the corresponding rates are shown in Fig. 1.18 for a typical serum. The optimal concentration appeared to vary with the activity of the serum, perhaps due to variable

EFFECT OF VARIATION IN ASSAY CONDITIONS ON THE REACTION RATE OF ALD IN HORSE SERUM

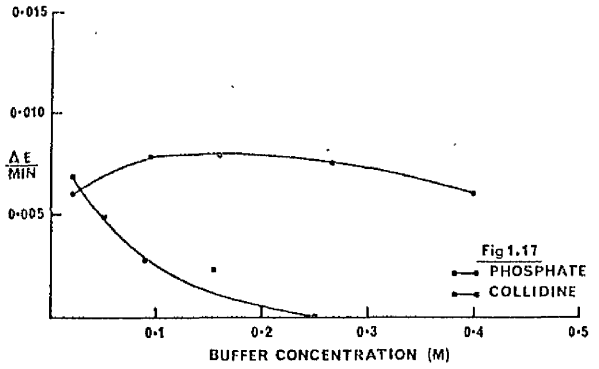


Fig 1.17

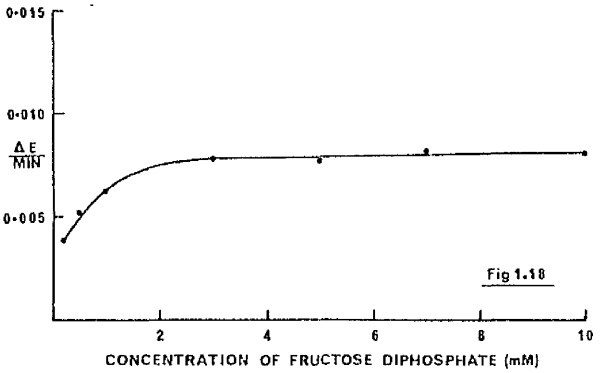


Fig 1.18

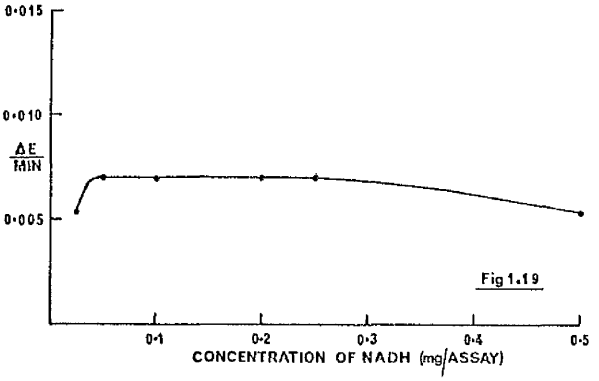


Fig 1.19

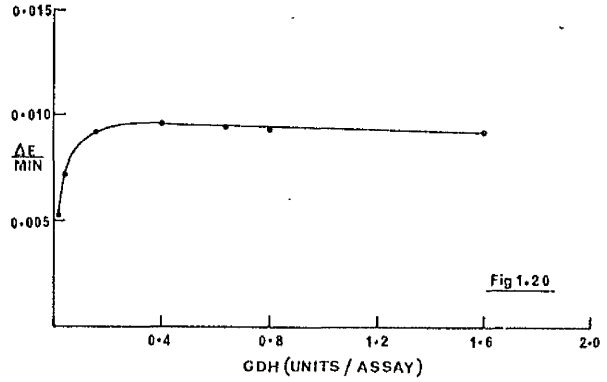


Fig 1.20

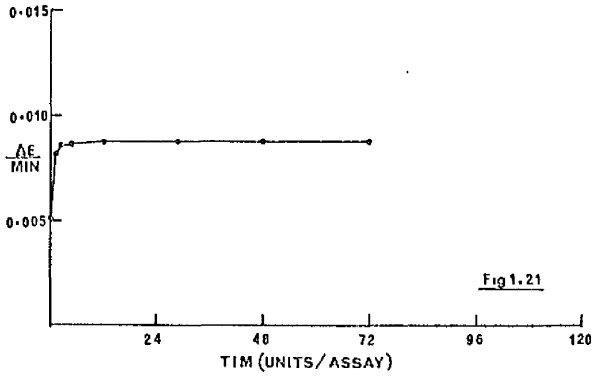


Fig 1.21

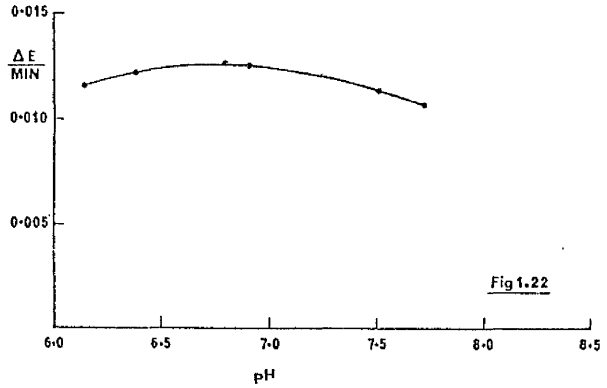


Fig 1.22

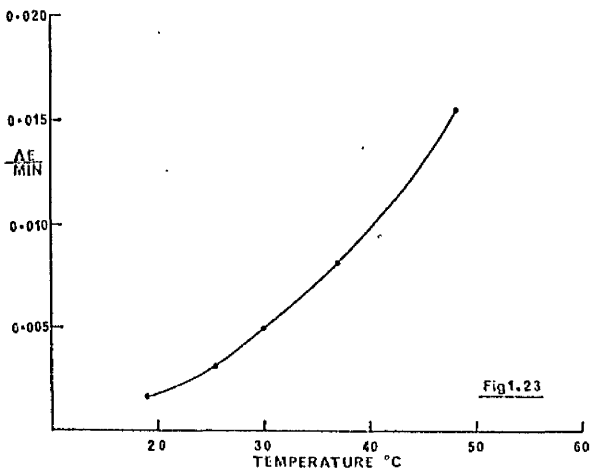


Fig 1.23

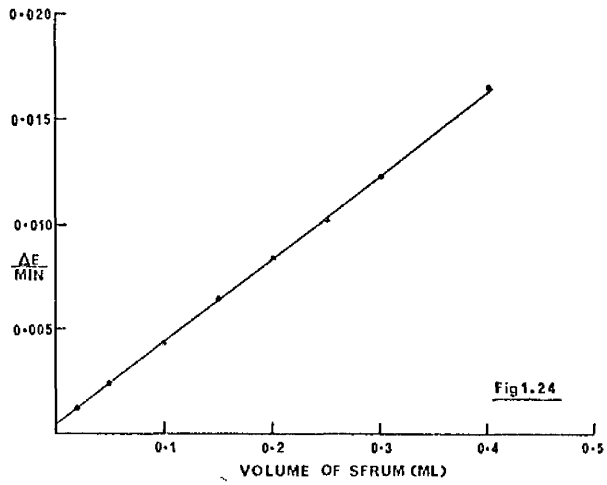


Fig 1.24

isoenzyme composition. The concentration used in all subsequent assays was 5 mM.

The Km (fructose-1,6-diphosphate) was calculated from a Lineweaver-Burk (1934) plot to be $2.6 \times 10^{-4} \pm 1.3 \times 10^{-4}$M.

(c) Variation in rate with iodoacetate concentration

In some aldolase assay systems (Bruns, 1954), iodoacetate is included in the medium to inhibit possible glyceraldehyde-3-phosphate dehydrogenase activity, since the presence of this enzyme would interfere with the aldolase assay. In this work, however, the addition of iodoacetate was found to have no effect on aldolase activity, the activity being constant over the range 0-0.66 mM-iodoacetate. Despite this, to make comparison with other aldolase assay methods easier, a concentration of 0.3 mM was used in all subsequent assays.

(d) Variation in rate with NADH concentration

Fig. 1.19 illustrates the effect on a typical serum of varying the concentration of NADH in the assay medium. The optimal concentration was taken as 0.25 mg/NADH/assay (0.12 mM) with marked inhibition at 0.50 mg/assay (0.24 mM).

(e) Variation in rate with glycerol phosphate dehydrogenase (GDH) concentration

Aldolase activity increased with increasing concentration of GDH up to a maximum around 0.3 units added GDH. At this concentration, there was sufficient auxiliary enzyme present to

reduce all the dihydroxyacetone phosphate as soon as it was formed in the aldolase reaction. Increasing the GDH concentration above 0.4 units did not increase the reaction rate and some inhibition was seen at 1.6 units/assay for the serum in Fig. 1.20. In all subsequent assays 0.4 units GDH were added to the assay mixture.

(f) Variation in rate with triose phosphate isomerase (TIM) concentration

Optimal activity was obtained with 12 units of added TIM (results for a typical serum are shown in Fig. 1.21) and this concentration was used in all subsequent assays. (With no added TIM the reaction still proceeded although at a suboptimal rate).

(g) Variation in rate with pH

Fig. 1.22 illustrates the effect of varying the pH of the assay mixture in the range 6.15-7.75. Horse aldolase activity was optimal at pH 6.5-7.0 with consistently lower activity at pH 7.4 for all sera studied.

(h) Variation in rate with temperature

The temperature of the reaction was varied over the range 19-48^{o}C and the reaction rate increased with increasing temperature within this range (Fig. 1.23). All subsequent assays were carried out at 37^{o}C. (There was no detectable thermal denaturation at 37^{o}C even after a 45 minute incubation). Arrhenius plots were made using the reaction rates at 19, 25.5, 30 and 37^{o}C. The mean slope of these plots was -3517 ± 206. Using this value,

factors were calculated to convert the activity at other commonly used temperatures to that at 37°C. These are shown in Table I.I.

(i) Variation in rate with enzyme concentration

The reaction rate is shown in Fig. 1.24 (for a typical serum) to be proportional to the enzyme concentration (varied by varying the volume of serum in the reaction mixture from 0.02 to 0.40 ml.). In this instance, the activity had to be corrected for the "blank" activity resulting from the spontaneous degradation of NADH and from the oxidation of NADH by side reactions. When this is done, the line in Fig. 1.24 passes through zero.

Optimal Conditions for the Assay of Horse Serum Aldolase

As a result of the above studies, the final conditions chosen for the assay of aldolase in horse serum were:-
5 mM-fructose-1,6-diphosphate, 0.3 mM-iodoacetate, 0.25 mg. NADH/assay (0.12 mM), 0.4 units GDH/assay and 12 units TIM/assay in 0.1M-phosphate buffer, pH 7.0 at 37°C. The final volume was 3.0 ml.

Reagents

1M and 0.1M-phosphate buffer, pH 7.0:- as for LDH.

0.15M-fructose-1,6-diphosphate in 0.1M-buffer, pH 7.0:-

0.609 g. fructose-1,6-diphosphate were dissolved in 10 ml. 0.1M-phosphate buffer, pH 7.0 and the pH checked. (Stored at -15°C).

0.01M-iodoacetate in 0.1M buffer, pH 7.0:- 37.2 mg. iodoacetic acid were dissolved in 2 ml. 1.0M-phosphate buffer and approximately 12 ml. distilled water. The pH was adjusted to 7.0 with 1M-NaOH and the solution made up to 20 ml. (Stored at -15°C).

3.6 mM-NADH solution:- Sufficient NADH to give a 2.5 mg/ml. solution was dissolved in 0.1M-phosphate, pH 7.0. (Made up fresh daily).

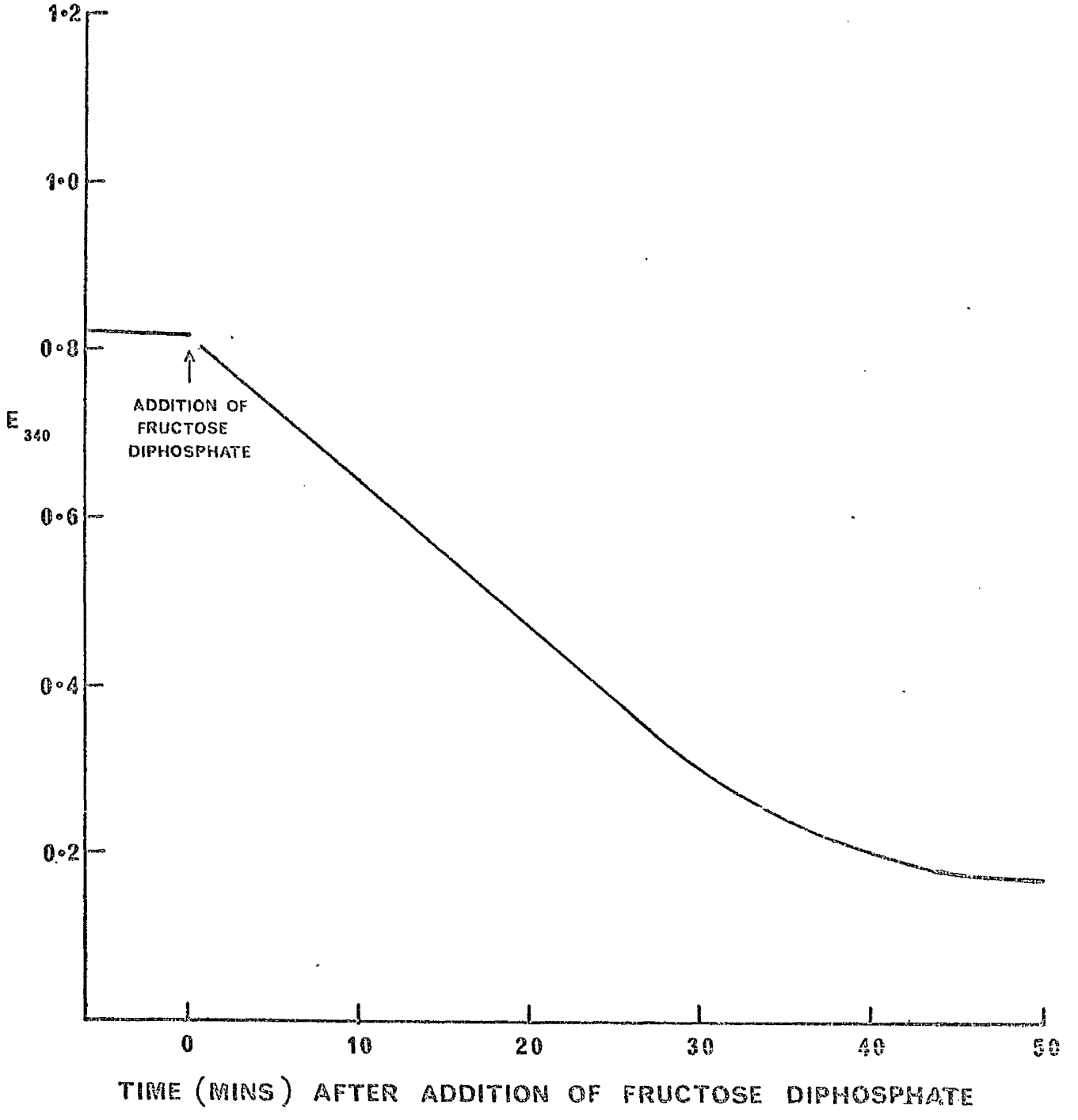
GDH solution (4 units/ml.):- Stock solution was diluted with 0.1M-phosphate, pH 7.0 to give a solution containing 4 units GDH/ml. (Made up fresh daily).

TIM solution (120 units/ml.):- Stock solution was diluted as above to give a solution containing 120 units TIM/ml. (Made up fresh daily).

Assay procedure

2.3 ml. phosphate buffer, 0.1 ml. iodoacetate solution, 0.1 ml. NADH solution, 0.1 ml. TIM solution, 0.1 ml. GDH solution and 0.2 ml. serum were added to a quartz cuvette. After mixing, the solution was incubated in the spectrophotometer at 37°C for 5 minutes. At the end of this time extinction readings were recorded for 2-3 minutes. This gave the "blank" activity for the serum. The reaction was started by the addition of 0.1 ml. fructose-1,6-diphosphate solution (previously warmed to 37°C), the solution mixed and extinction readings recorded for 5-10 minutes.

Fig 1.25 REACTION PROGRESS CURVE FOR ALDOLASE



Because of the relatively high "blank" activity, the ΔE/min. for the incubation period was subtracted from the overall ΔE/min. The aldolase activity was calculated from the resulting ΔE/min., taking into account the fact that two molecules of NADH are oxidised for every molecule of fructose diphosphate transformed. Therefore the R.H.S. of equation (1) was divided by a factor of 2.

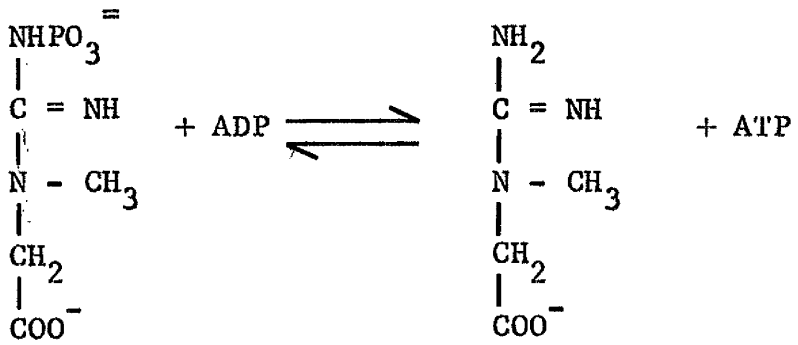
Using this method, the reaction was linear for at least 20 minutes. A diagrammatic representation of the reaction progress curve of horse aldolase is shown in Fig. 1.25.

Reproducibility of the method

Of 112 assays performed in duplicate the mean difference between duplicates was found to be 0.6 mI.U./ml. The mean of 6 replicate measurements made on one serum was 11.7 $\pm$ 0.8 mI.U./ml. with a coefficient of variation of 6.5%. Daily assays performed on frozen aliquots of the same serum for 4 days gave a mean of 16.5 $\pm$ 0.5 mI.U./ml. with a coefficient of variation of 2.9%.

(4) Determination of Optimal Working Conditions of Horse Serum Creatine Kinase (CK) - E.C.2.7.3.2

Principle of assay - Creatine kinase catalyses the reversible formation of adenosine triphosphate (ATP) and creatine from adenosine diphosphate (ADP) and creatine phosphate i.e.



creatine phosphate creatine

In this work, the reaction is assayed in the forward direction, i.e. from creatine phosphate to creatine, since the rate of the forward reaction is several times greater than that of the reverse under optimal conditions (King, 1965). The method is based on the spectrophotometric procedure for CK developed by Oliver (1955) and using the following series of reactions:-

Creatine phosphate + ADP $\rightleftharpoons$ creatine + ATP

ATP + glucose $\xrightleftharpoons{HK}$ glucose-6-phosphate + ADP

Glucose-6-phosphate + $NADP^+$ $\xrightleftharpoons{GPDH}$ 6-phosphogluconate + NADPH.

Provided glucose, nicotinamide adenine dinucleotide phosphate ($NADP^+$), hexokinase (HK) and glucose-6-phosphate dehydrogenase (GPDH) are added in excess, the rate of reduction of $NADP^+$ (the rate of increase of E_{340}) is proportional to the CK activity.

The effects of varying the conditions of assay are discussed below.

(a) Variation in rate with creatine phosphate concentration

The initial conditions used were 20 mM-glucose, 10 mM-

magnesium acetate, 10 mM-AMP, 1 mM-ADP, 1.5 mg. $NADP^+$/assay (0.6 mM), 7 units HK/assay, 2.5 units GPDH/assay, 10 mM-glutathione and 0.2 ml. serum in 0.1M-triethanolamine buffer, pH 7.0. Fig. 1.26 shows the effect of varying the concentration of phosphocreatine in the assay mixture for a typical serum. A concentration of 16.7 mM was chosen for use in all subsequent assays. The Michaelis constant Km (creatine phosphate) was estimated to be $1.3 \times 10^{-3} \pm 0.5 \times 10^{-3}$M.

(b) Variation in rate with ADP concentration

Fig. 1.27 shows that horse CK activity was optimal at 1-2 mM-ADP concentration and in all subsequent assays 1 mM-ADP was used. The Km (ADP) was calculated to be $3.2 \times 10^{-4} \pm 1.3 \times 10^{-4}$M.

(c) Variation in rate with glucose concentration

A glucose concentration of 20 mM gave optimal CK activity and this concentration was used in all subsequent assays. Fig. 1.28 shows the results for a typical serum.

(d) Variation in rate with magnesium (Mg^{++}) concentration

Magnesium was required as a metal cofactor in the kinase reaction. Optimal rates were obtained at a Mg^{++} concentration of 10 mM (Fig. 1.29) with considerable inhibition at higher concentrations.

(e) Variation in rate with AMP concentration

AMP was added to the reaction mixture to inhibit myokinase (a muscle enzyme which produces ATP and AMP from two molecules of ADP). Fig. 1.30 shows the effect on the reaction rate of varying the concentration of this material for a typical serum. Increasing

EFFECT OF VARIATION IN ASSAY CONDITIONS ON THE REACTION RATE OF CK IN HORSE SERUM

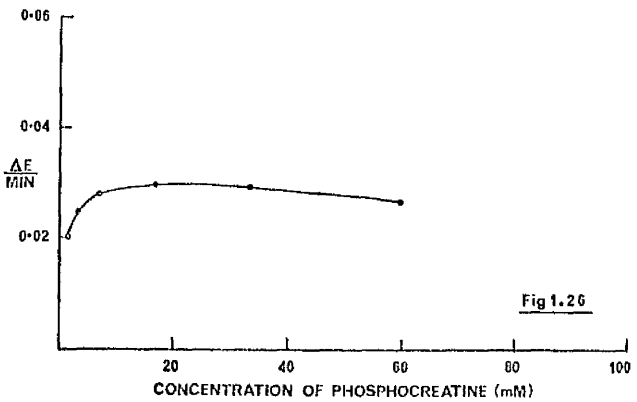


Fig 1.26

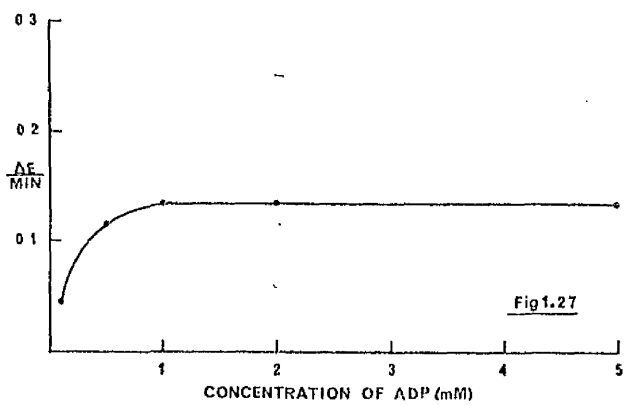


Fig 1.27

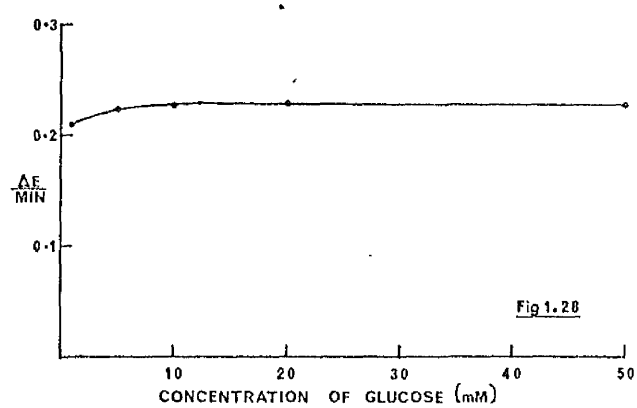


Fig 1.28

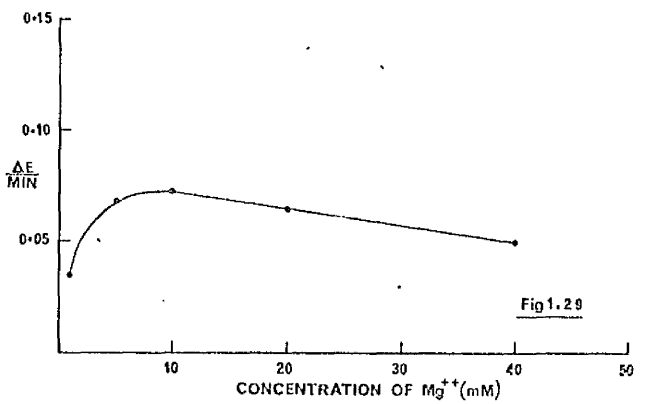


Fig 1.29

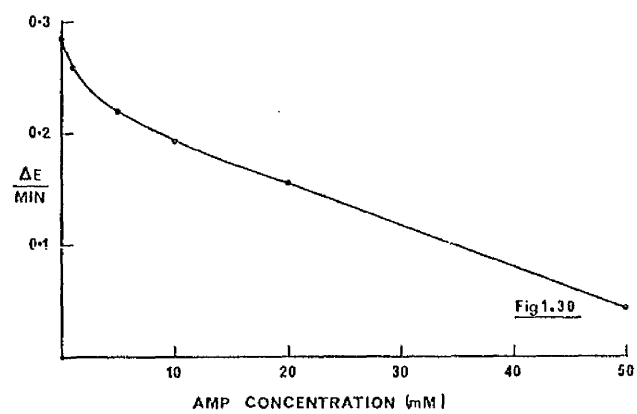


Fig 1.30

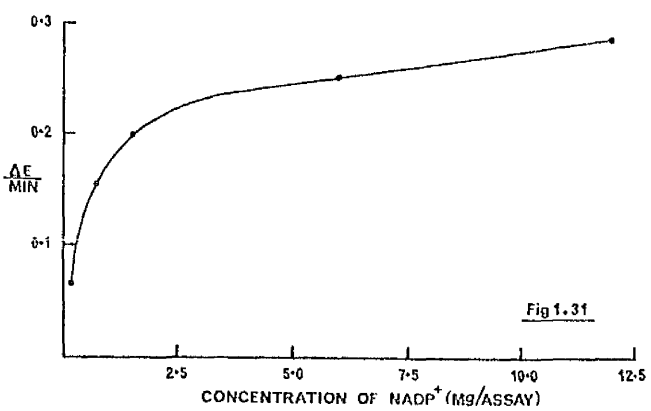


Fig 1.31

the concentration of AMP decreased the reaction rate, the decrease being greater at low concentrations. In all subsequent assays 10 mM-AMP was used.

(f) Variation in rate with $NADP^+$ concentration

Although the reaction rate was still increasing at 12 mg/assay (4.8 mM) in the case shown in Fig. 1.31, in all subsequent assays 4 mg. $NADP^+$/assay (1.6 mM) were used. The 10% increase in rate obtained by increasing the $NADP^+$ concentration from 4 to 8 mg/assay did not justify the cost of the extra material.

(g) Variation in rate with glutathione concentration

The addition of a thiol compound, in this case glutathione, to the reaction mixture increased the CK activity. Fig. 1.32 shows that 5 mM-glutathione reactivated the enzyme completely. In all subsequent assays 10 mM-glutathione was used.

(h) Variation in rate with hexokinase concentration

The minimum concentration of hexokinase required for optimal activity was 2.5 units/assay as shown in Fig. 1.33 for a typical serum and this concentration was used throughout this work.

(i) Variation in rate with GPDH concentration

Fig. 1.34 illustrates the variation in rate with GPDH concentration. 4 units/assay were chosen as a suitable concentration for subsequent assays although some high activity sera showed a slowly increasing rate even at 6 units/assay.

EFFECT OF VARIATION IN ASSAY CONDITIONS ON THE REACTION RATE OF CK IN HORSE SERUM

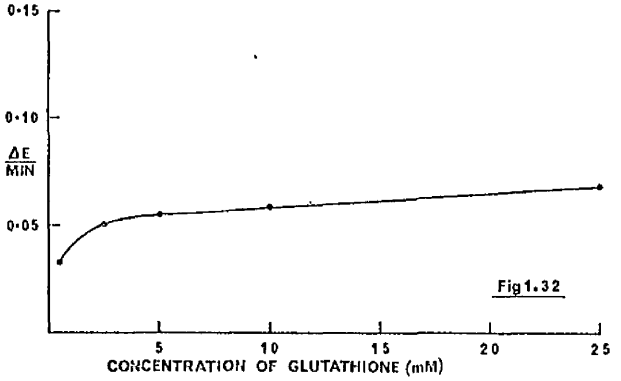


Fig 1.32

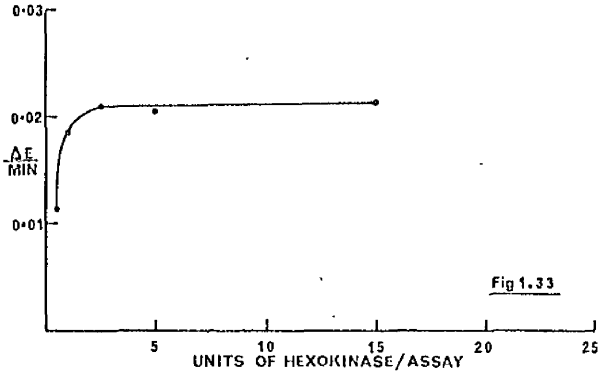


Fig 1.33

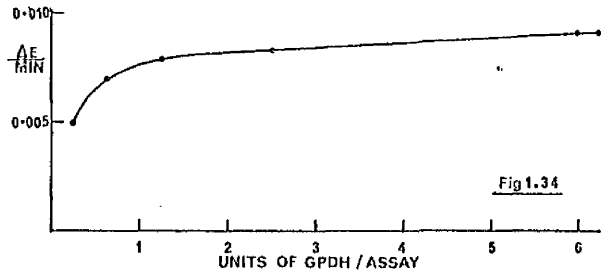


Fig 1.34

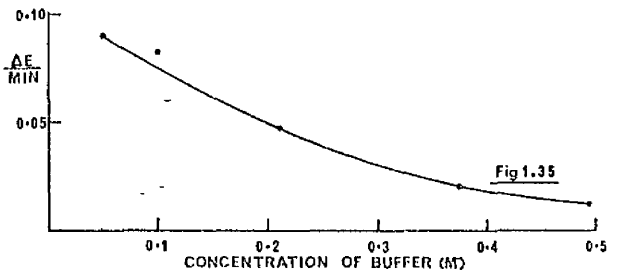


Fig 1.35

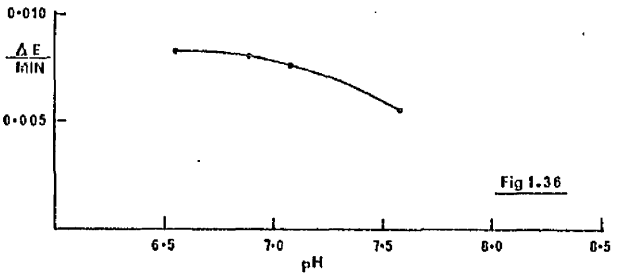


Fig 1.36

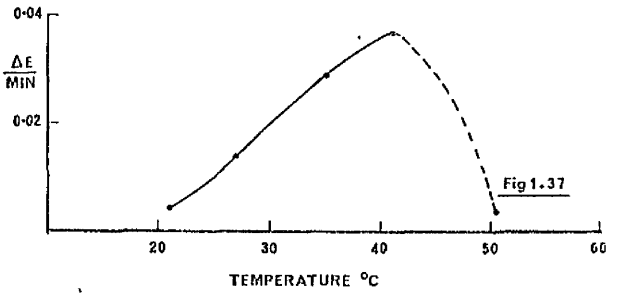


Fig 1.37

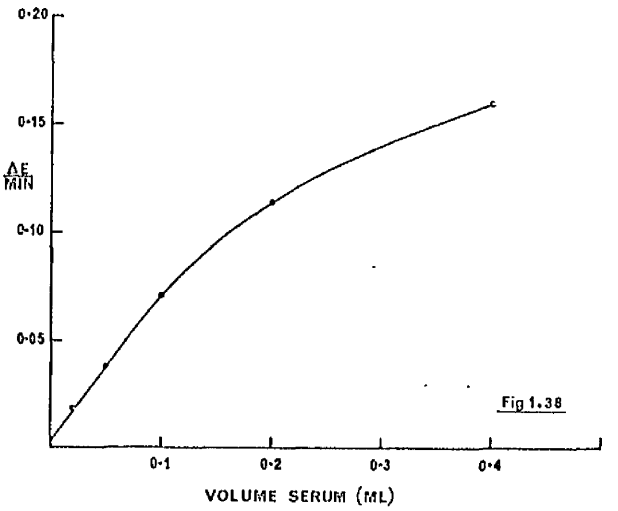


Fig 1.38

(j) Variation in rate with buffer concentration

The reaction rate decreased with increasing concentration of triethanolamine buffer, pH 7.0. (Fig. 1.35 shows the result for a typical serum). A 0.1M buffer was used in all subsequent assays.

(k) Variation in rate with pH

Fig. 1.36 shows that the reaction rate decreased with increasing pH over the range studied i.e. from pH 6.5-7.5. As a result all subsequent assays were carried out at pH 7.0.

(l) Variation in rate with temperature

The effect of temperature on horse serum CK activity was investigated. The rate increased with increasing temperature in the range 21-41°C. The activity at 50.5°C was considerably less than that at 41°C (the effect on a typical serum is shown in Fig. 1.37), indicating thermal denaturation at the higher temperature. Incubation at 37°C, however, even for an extended incubation period of 45 minutes did not result in any detectable loss of activity and 37°C was the temperature chosen for all subsequent assays. Using the rates at 21, 27 and 35°C, Arrhenius plots were constructed and the mean slope of these plots calculated to be -3730 ± 269. From this value, factors to convert CK activity at other temperatures to that at 37°C were calculated (shown in Table I.I).

(m) Variation in rate with enzyme concentration

The relationship between activity and enzyme concentration was seen to be non-linear (Fig. 1.38), the rates at high enzyme concentrations being less than expected. In all subsequent assays 0.1 ml. serum was used since the deviation from linearity was more pronounced with serum volumes greater than 0.1 ml.

Optimal Conditions for the Assay of Horse Serum CK

As a result of the above studies, the final conditions chosen for the assay of CK in horse serum were: 16.7 mM-creatine phosphate, 20 mM-glucose, 10 mM-magnesium acetate, 10 mM-AMP, 1 mM-ADP, 4 mg. NADP/assay (1.6 mM), 10 mM-glutathione, 2.5 units HK/assay and 4 units GPDH/assay in 0.1 M-triethanolamine buffer, pH 7.0 at 37°C. The final volume was 3.0 ml.

Reagents

1M-triethanolamine-HCl buffer, pH 7.0:- 185.6 g. triethanolamine-HCl were dissolved in approximately 600 ml. distilled water, the pH adjusted to 7.0 with 4 M-NaOH and the volume made up to 1 litre.

0.1M-triethanolamine buffer, pH 7.0:- Diluted from 1 M buffer with distilled water and the pH checked.

0.05 M-creatine phosphate in 0.1 M buffer:- 1.816 g. creatine phosphate (sodium salt) were dissolved in 80 ml. distilled water and 10 ml. 1M-triethanolamine buffer. The pH was

adjusted to 7.0, if necessary, with 1M-NaOH, and the solution made up to 100 ml. (Stored frozen at -15°C).

0.3M-glucose in 0.1 M buffer:- 2.702 g. glucose were dissolved in 50 ml. 0.1 M buffer. (Stored frozen at -15°C).

0.3M-magnesium acetate in 0.1 M buffer:- 1.287 g. magnesium acetate were dissolved in 20 ml. 0.1 M buffer and the pH checked. (Stored frozen at -15°C).

1.6mM-$NADP^+$ solution:- Sufficient $NADP^+$ (corrected for purity) to give a solution of 40 mg/ml was dissolved in 0.1 M buffer. (Made up fresh daily).

0.3M-AMP solution:- 2.084 g. AMP were dissolved in approximately 15 ml. distilled water and 2 ml. 1 M buffer. The pH was adjusted to 7.0 and the volume made up to 20 ml. (Stored frozen at -15°C).

0.15M-glutathione in 0.1 M buffer:- 0.922 g. reduced glutathione were dissolved in 2 ml. 1 M buffer and 15 ml. water. The pH was adjusted to 7.0 and the volume made up to 20 ml. (Stored frozen at -15°C).

0.03M-ADP in 0.1 M buffer:- 0.304 g. ADP (sodium salt) were dissolved in 2 ml. 1 M buffer and 15 ml. water. After adjusting the pH to 7.0, the volume was made up to 20 ml. (Stored frozen at -15°C).

Hexokinase solution (25 Units/ml):- Diluted from the stock solution with 0.1 M buffer, pH 7.0, to give a solution

containing 25 Units/ml. (Made up fresh daily).

Glucose-6-phosphate dehydrogenase solution (40 Units/ml):- Diluted from the stock solution to give a concentration of 40 Units/ml. (Made up fresh daily).

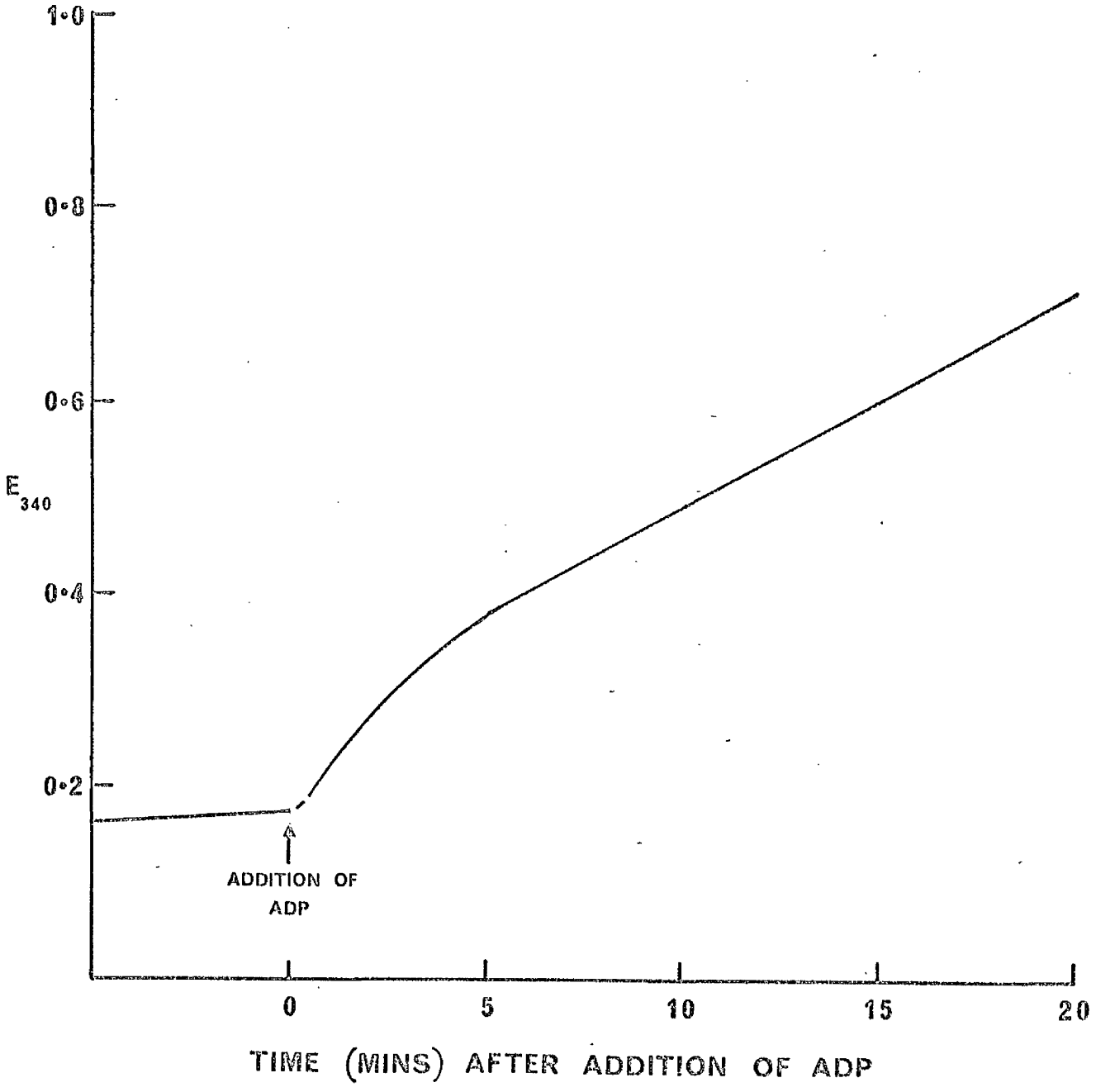
Assay procedure

0.9 ml. 0.1M-triethanolamine buffer, pH 7.0, 1.0 ml. 0.05 M-creatine phosphate solution, 0.2 ml. 0.3 M-glucose solution, 0.1 ml. 0.3 M-magnesium acetate solution, 0.1 ml. $NADP^+$ solution, 0.1 ml. 0.3 M-AMP solution, 0.2 ml. 0.15 M-glutathione solution, 0.1 ml. hexokinase solution, 0.1 ml. GDPH solution and 0.1 ml. serum were added to a quartz cuvette and mixed. After incubation in the spectrophotometer at 37^oC for 5 minutes, the reaction was started by the addition of 0.1 ml. ADP solution (previously warmed to 37^oC.) The solution was again mixed and after an interval of 5 to 6 minutes extinction readings were recorded for a further 5 minutes. The CK activity of the serum was calculated from the $\Delta E/min$, no blank being necessary using this method.

Linearity of reaction

Fig. 1.39 shows a diagrammatic representation of the reaction progress curve for CK. Following the addition of ADP to the assay medium a high initial rate of reaction was observed but this decreased within 5 minutes to give a rate which was linear

Fig 1.39 REACTION PROGRESS CURVE FOR CK



for at least 10 minutes. This high initial rate was due largely to myokinase since it was still observed in the absence of creatine phosphate but was abolished by the inclusion of excess AMP. The myokinase reaction ceased, however, within a few minutes and CK activity could be measured without the use of a blank. For this reason the ΔE/min. was not measured until 5 minutes after the start of the reaction.

Reproducibility of the method

Using the "Modified Method for CK" the reproducibility of the method was tested. When 25 samples were assayed in duplicate, the mean difference between duplicates was calculated to be 2.9 mI.U/ml. The mean of 6 replicate measurements made on a serum was found to be 140.4 $\pm$ 1.8 mI.U/ml. with a coefficient of variation of 1.3%.

The variation of the method from day to day was estimated by assaying frozen aliquots of the same serum on 5 consecutive days. This gave a mean of 138.5 $\pm$ 4.1 mI.U/ml. (coefficient of variation 2.9%).

DISCUSSION

The interpretation of kinetic studies on serum enzymes is complicated by various features of the assay material. First of all, the enzymes are not purified and are being assayed in a medium, serum, which may contain numerous interfering activators or inhibitors. Second, the enzyme activity of the serum may be made up of several isoenzymes catalysing the same reaction but obeying very different kinetics, e.g. the well documented instance of the pH optima of LDH isoenzymes. The final choice of assay conditions is dictated by the isoenzyme composition of the serum. This composition, however, may vary under different circumstances, e.g. in disease states, after exercise, etc. and the assay conditions chosen must, of necessity, be a compromise. With this in mind, the kinetic studies described have been carried out on sera from different horses covering a wide range of activities and, presumably, isoenzyme compositions.

The results of these studies indicate that several differences exist between the optimal conditions for assay of these enzymes in the horse and in man.

Horse serum glutamic-oxalacetic transaminase

This enzyme requires higher concentrations of aspartate and α-oxoglutarate than those quoted for human GOT. Previous

reports have given optimal concentrations of aspartate as 33 mM (Karmen, 1955), 46 mM (Laursen, 1959) and 125 mM (Henry, Chiamori, Golub and Berkman, 1960). In the light of the above investigations, however, all of these concentrations would be sub-optimal for the GOT found in horse serum. Similarly the 10 mM concentration of α-oxoglutarate found here is slightly higher than the 6.7 mM used in the method of Karmen (1955) but in keeping with the 11.6 mM reported as optimal for human GOT by Laursen (1959).

The pH optimum for human serum GOT has previously been reported as 7.4 (Nisonoff and Barnes, 1952; Steinberg, Baldwin and Ostrow, 1956) which is in good agreement with the present findings for horse serum GOT.

In most of the methods currently in use for enzyme assays in clinical laboratories the assay temperatures range from 25° to 37°C. The original temperature recommended by the Enzyme Commission (1961) for enzyme assays was 25°C but this was later amended to 30°C when it became obvious that the cell compartments of most spectrophotometers could only be maintained at 25°C with some difficulty (the use of 30°C eliminated this problem in most climates). Even at 30°C, however, the estimation of some enzymes, such as aldolase, which are present in serum in very low concentrations, is difficult. To increase the accuracy of

such assays, the temperature is increased here to 37°C. Since thermal denaturation during incubation at 37°C has been shown above to be negligible for all four enzymes under study, this temperature is also used for GOT, LDH and CK assays throughout this work. Because the slope of the Arrhenius plot (-2515) was found to be very close to that quoted for human GOT i.e. -2570 (Henry et al, 1960) and -2510 (Schneider and Willis, 1958) the temperature conversion factors for horse GOT (Table I.I) are almost identical to those in current use for the human enzyme.

Horse serum lactic dehydrogenase

The decrease in activity with increasing phosphate buffer concentration observed by Henry et al (1960) for human LDH was not demonstrated here. Horse serum LDH shows optimal activity at phosphate concentrations around 0.1-0.3M. This difference may be due to species variation since Hyldgaard-Jensen (1971), in studies on porcine LDH, found a peak of activity around 0.1M-phosphate when the enzyme was assayed from lactate to pyruvate.

Henry et al (1960) observed maximal activity for human LDH at a pyruvate concentration of approximately 0.6 mM with inhibition at 1.2 mM, while the horse enzyme has an optimum at 0.8-1.0 pyruvate with some inhibition at 3 mM. Studies on lactic dehydrogenases from other sources demonstrate variations in optimal pyruvate concentrations with species. Hyldgaard-Jensen (1971) reports optimal concentrations for porcine LDH_1

and LDH_5 as 0.6 mM and 2.1 mM respectively while bovine LDH_1 and LDH_5 have optima at 0.6 mM and 3.0 mM (Fondy and Kaplan, 1965).

The main objection put forward by Thiers and Vallee (1958) to assaying LDH in the direction pyruvate to lactate was the narrow optimal concentration of pyruvate and NADH and the inhibition caused by both these reactants when in excess of the optimum. Since pyruvate and NADH concentrations appear to be less critical for horse LDH, this objection is invalid for the horse.

Although the pH optimum for horse serum LDH has been taken as 7.0, the actual optimum varies with different sera. This can be explained in terms of the variable isoenzyme content of sera. The pH optima of human LDH_1 and LDH_5 have been given as 8.0 and 7.25 respectively for the pyruvate to lactate reaction (Clausen and Øvlisen, 1965). For the porcine isoenzymes, LDH_1 activity is constant over the range 5.0 to 7.4 while LDH_5 activity is at a maximum at pH 6.1 with a decline in activity above 7.0 (Hyldgaard-Jensen, 1971). The results reported above suggest that the horse enzyme resembles porcine LDH more than human LDH in its pH optimum.

The temperature conversion factors for LDH in Table I.I are in reasonably good agreement with the results of Laursen (1959) for human LDH but are lower than those quoted by Henry et al (1960) and King (1965).

Horse serum aldolase

Horse serum aldolase appears to require a higher concentration, 5 mM, of fructose-1,6-diphosphate than human aldolase although the optimum varies with the activity of the serum, perhaps due to variable isoenzyme composition. In the Boehringer Test Kit Method the concentration of FDP used is 2.7 mM which would be suboptimal for horse aldolase. Similarly, the 0.36 mM concentration of NADH used in the kit method would prove inhibitory to the horse enzyme since the optimum, determined above, is at 0.25 mg/assay (0.12 mM) with marked inhibition at 0.50 mg/assay (0.24 mM).

The pH optimum, 6.5-7.0, found here for horse aldolase is in contrast to data given for serum aldolase in the human. King (1965) reports that human liver aldolase shows little dependence on pH in the range 7.0-9.5 although the muscle enzyme has a lower optimum. The lower optimum found here for horse serum aldolase may either be due to a species difference or to a predominance of the muscle enzyme (if King's information on human aldolase can be extrapolated to the horse enzyme).

Horse serum creatine kinase

The concentrations of glucose, magnesium, ADP, AMP and glutathione providing optimal activity above for horse CK are in agreement with those used in the Boehringer Test Kit UV-Method (1969) which is based on the method of Oliver (1955).

The conditions in the methods differ only in the concentrations of creatine phosphate and $NADP^+$. The 16.7 mM concentration of creatine phosphate used here is lower than that used in the Boehringer Method, 35 mM, but higher than the 10 mM used in the original method of Oliver (1955). Also in the Kit Method, the concentration of $NADP^+$ is 0.6 mM which would be suboptimal for the horse enzyme on the basis of the above results.

Most methods based on the forward reaction i.e. from creatine phosphate to creatine, have been carried out in the pH range 7.0-7.4 for human CK (Oliver, 1955; Hughes, 1962). The pH used in the "Modified Method" above is 7.0 although the results indicate that the optimum for the horse enzyme may be lower than that for the human.

The temperature conversion factors for CK in Table I.I are slightly higher than those quoted for human CK by Rosalki (1967).

Whereas GOT, LDH and aldolase all show linear relationships between reaction rate and enzyme concentration, the CK results indicate some inhibition with high volumes of serum. This is suggested to be due to the presence in the serum of some inhibitor which is 'diluted out' at lower concentrations (Dixon and Webb, 1958).

In conclusion, therefore, these kinetic studies demonstrate that some methods in widespread use in clinical enzymology may not be ideal for use in the veterinary field. The variations in

optimal working conditions have been discussed here for some enzymes in horse serum. Similar variations may, and most probably do, exist for other species.

Results obtained using commercial kits may only be approximate for species other than the human since the kit manufacturers use assay conditions which are optimal for the largest section of their market i.e. for human medicine. For routine diagnostic purposes an approximation of this sort will generally be sufficient since the changes in serum enzyme levels with disease are usually marked. In research, however, where the changes being measured may be very small, care should be taken to ensure that the method chosen uses optimal working conditions for the enzyme under study.

SECTION II

Studies on the Effects of Exercise on Serum Enzyme Levels in the Horse

In this section the normal levels of GOT, LDH, ALD and CK in horse serum are established together with the individual variation noted in these values. The effects of exercise and of training on the enzyme levels are also investigated.

MATERIALS AND METHODS

Materials

The chemicals and enzymes used are listed in the previous section with the addition of Heparin (Evans Medical Ltd., Speke, Liverpool).

Animals

The horses used in this study consisted of a group of clinically normal mares and geldings of both hunter and thoroughbred types - a brief description of the animals (sex, age, etc.) is given in Table 2.1.

Daily maintenance routine

The animals were housed in loose boxes with straw for bedding. If, however, any animal began to eat the straw, this was replaced by wood shavings. Each animal was maintained on a strict diet consisting of 16 kg. hay, 0.5 kg. bruised oats, 1.5 kg. bran, 2 kg. pony cubes and 20 ml. treacle per day. This was divided into two meals, one at 09.30 hours and the other at 16.30 hours. Daily maintenance exercise was rigidly controlled and

TABLE 2.1 DESCRIPTION OF ANIMALS USED

Horse No.	Type	Sex	Age (Years)
1	Hunter	G	8
2	T/B	G	9
3	Hunter	F	9
4	T/B	G	8
5	Pony	F	6
6	T/B	F	13
7	T/B	G	Aged
8	Pony	G	Aged
9	Pony	G	1
10	Pony	G	10
11	Hunter	G	9
12	T/B	G	5
13	Hunter	G	9
14	Hunter	G	10
15	T/B	G	15
16	Pony	F	3
17	T/B	G	7
18	Hunter	G	Aged

T/B = Thoroughbred, G = Gelding and F = Female

consisted of trotting for 20-30 minutes. Sampling was carried out at the same time each day i.e. after the morning feed and before the animals were exercised.

Experimental exercise

Three experimental exercise programmes were used, made up as follows:

Exercise A = 8 Km trot + 4 Km canter + 8 Km gallop = 20 Km

Exercise B = 4 Km trot + 2 Km canter + 4 Km gallop = 10 Km

Exercise C = 4 Km trot + 6 Km canter = 10 Km

All exercises were carried out in a large field between 10.00 hours and 12.00 hours. On days when the animal was subjected to one of these exercise programmes, the daily maintenance exercise was omitted.

Methods

Collection and preparation of blood from the horse for enzyme analyses

Serum was obtained as described previously (Section I). When plasma was required blood was collected in a heparinised polystyrene tube (300 I.U. heparin/10 ml. blood), centrifuged at 1,000 g. for 5 minutes and the plasma removed and stored at -15°C (unless assays were to be carried out immediately).

Measurement of serum enzyme levels

Serum GOT, LDH, ALD and CK levels were measured by the methods described in the previous section using conditions which are optimal for the horse enzymes.

RESULTS

(1) The stability of GOT, LDH, ALD and CK in horse serum on storage

Fresh serum was divided into 1.5 ml. aliquots and these were stored at -15°C, 4°C and room temperature (mean temperature of 20°C). The enzyme activity at each temperature was determined at intervals over a period of 2 months. All assays were performed in duplicate. This was carried out on serum from four horses and typical results are shown in Fig. 2.1. GOT is seen to be the most stable of the four enzymes with only 8% loss of activity in 67 days at -15°C and 13% at 4°C. Storage at room temperature is unsatisfactory for all of the enzymes studied.

The effects of repeated freezing and thawing were also investigated, samples being frozen 12 times in 7 days. No detectable loss of activity was observed (Table 2.2) during this procedure.

(2) Comparison of the enzyme activities of serum and plasma

A comparison was made of the enzyme activities of horse serum and plasma. Several samples were taken from each of 3 horses on different occasions and the blood was immediately divided, some being collected in a plain tube and some in a heparinised tube. To eliminate any effect due to loss of activity in serum because of the time required for clotting, etc.,

Fig 2.1 STABILITY OF HORSE GOT, LDH, ALD AND CK AT −15°C, 4°C AND ROOM TEMPERATURE

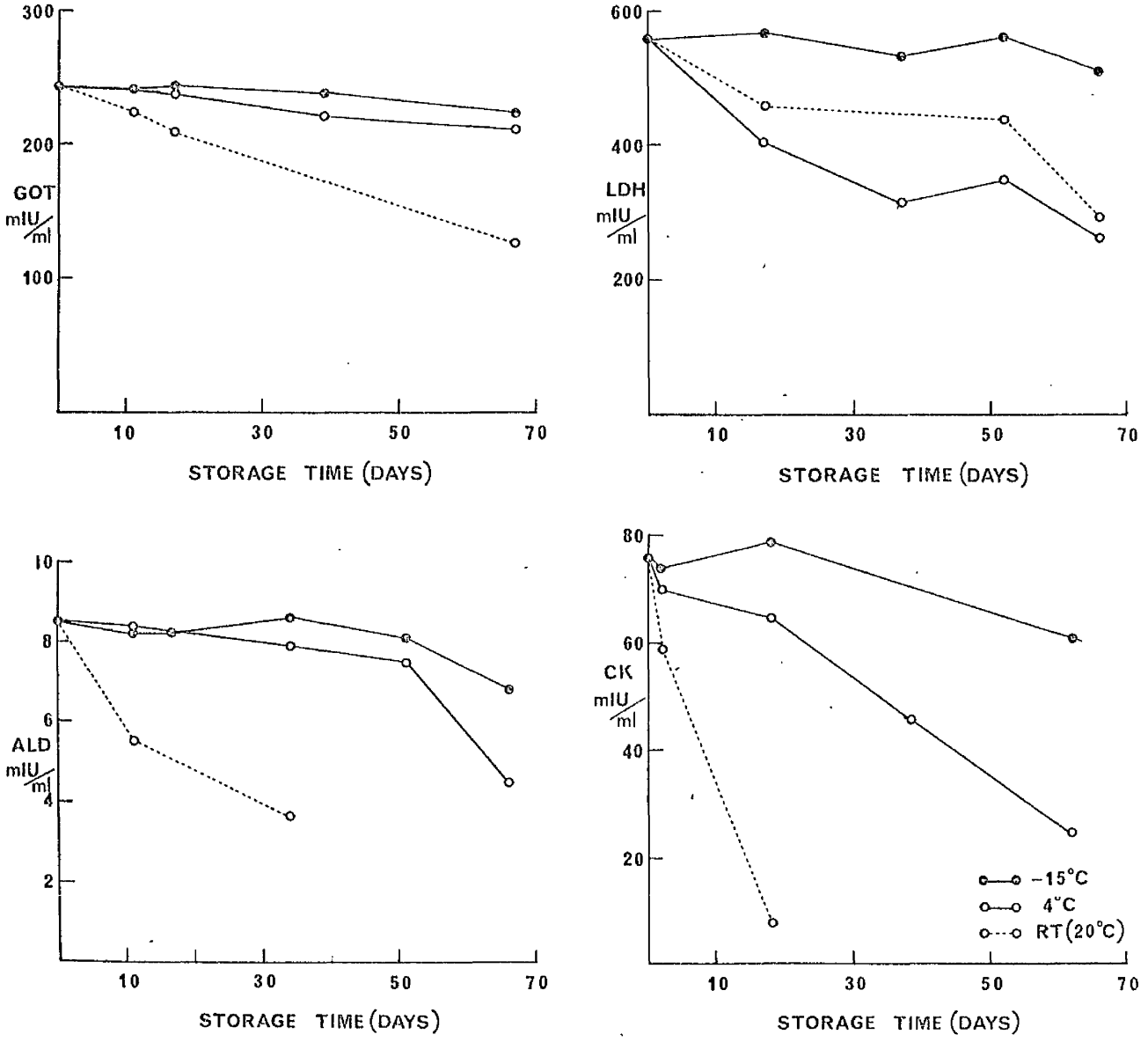


TABLE 2.2 EFFECT OF REPEATED FREEZING AND THAWING ON SERUM ENZYME ACTIVITY

No. of Times Frozen/ Thawed	Activity (mIU/ml) of Serum			
	GOT	LDH	ALD	CK
0	242	569	8.3	73
4	243	576	8.9	76
8	244	583	8.8	73
12	243	588	8.9	74

plasma samples were also kept at room temperature until the serum was ready. Both the serum and plasma were assayed for GOT, LDH, ALD and CK and the results are shown in Table 2.3. Analysis of these results by the Student's 't' test showed no significant difference between the activities in serum and plasma.

(3) Normal range for horses on a controlled diet and maintenance exercise

The normal range for serum levels of GOT, LDH, ALD and CK in a group of 10 horses is shown in Table 2.4a. Table 2.4b gives corresponding data from the literature for comparison. It will be seen that there was little variation in serum GOT activity between individuals of the same species but considerable variation in LDH, ALD and CK activity.

(4) Daily variation in serum enzyme levels

The variation in serum enzyme levels from day to day was determined by taking samples from each horse for 7-10 days prior to beginning an exercise programme. The results are shown expressed in Table 2.5 for 2 horses. The daily variation for each individual is seen to be less than that between individuals of the same species.

(5) Effect of experimental exercise on serum levels of GOT, LDH, ALD and CK

Four horses were subjected to exercise A, samples being

TABLE 2.3. COMPARISON OF GOT, LDH, ALD AND CK ACTIVITY OF SERUM AND PLASMA

Serum	GOT activity (mIU/ml)		LDH activity (mIU/ml)		ALD activity (mIU/ml)		CK activity (mIU/ml)	
	Plasma	Serum	Plasma	Serum	Plasma	Serum	Plasma	Serum
1	197	204	290	299	8.2	8.0	85	72
2	222	227	307	246	8.6	7.4	85	79
3	217	216	367	403	11.0	11.2	104	103
4	300	307	514	495	20.4	15.1	117	113
5	278	278	389	411	11.2	10.7	95	91
6	231	242	328	374	-	-	90	106
7	213	215	275	333	8.0	10.6	-	-
8	205	217	336	415	10.5	13.6	-	-
9	225	221	328	355	10.1	10.4	-	-
10	239	243	415	394	14.0	12.4	-	-
11	234	246	398	382	12.3	12.4	-	-
12	248	259	369	423	-	-	-	-
13	302	305	437	497	11.8	13.2	179	190
14	302	303	473	611	13.8	21.1	172	199
15	303	307	519	519	14.4	15.0	219	216
16	293	298	531	526	15.0	15.9	203	215
17	284	284	565	522	16.9	13.8	187	182

Sera 1-6 were obtained from Horse 2 on different occasions, Sera 7-12 from Horse 3 and sera 13-17 from Horse 12.

TABLE 2.4a. NORMAL RANGE OF SERUM ENZYME LEVELS IN THE HORSE

No. of Horses	Enzyme	Serum Activity (mIU/ml) Mean	S.D.	Range	Exercise
10	GOT	275	± 34	(235-339)	Routine trotting for 20-30 minutes daily
10	LDH	678	± 165	(432-944)	
10	ALD	12.4	± 4.0	(7.5-20.3)	
10	CK	111	± 33	(65-170)	

TABLE 2.4b. NORMAL RANGE FOR THE HORSE FROM DATA IN THE LITERATURE

No. of Horses	Enzyme	Serum Activity (mIU/ml) Mean	S.D.	Range	Exercise
43	GOT [a]	86	± 25	(48-171)	None
89	LDH [b]	374	± 136	(186-632)	No details
91	ALD [b]	15	± 3.4	(6-24)	No details
43	CK [a]	1.3	± 0.9	(0.0-3.6)	None

a From Cardinet, Littrell and Freedland (1967)

b From Gerber (1965a)

TABLE 2.5 DAILY VARIATION IN SERUM ENZYME LEVELS

Enzyme	Horse 1 Serum Activity (mIU/ml) Mean	S.D.	Range	Horse 6 Serum Activity (mIU/ml) Mean	S.D.	Range
GOT	326	± 17	(296-358)	250	± 16	(231-272)
LDH	625	± 24	(543-660)	446	± 59	(357-493)
ALD	7.5	± 0.5	(6.8-8.3)	7.8	± 1.4	(6.3-10.0)
CK	73	± 7	(58-84)	60	± 13	(42-75)

taken before, during and at intervals for 72 hours after the end of the exercise. The samples were assayed for GOT, LDH, ALD and CK and the results are shown for one horse in Fig. 2.2. Serum LDH and ALD began to rise during exercise and reached peak levels approximately 24 hours after the exercise had ceased. These enzymes remained elevated for 3-4 days. CK became elevated following exercise with peak levels around 5-6 hours after exercise and returned to pre-exercise levels within 2 days. Although a small but consistent increase in serum GOT activity was seen this remained within the daily variation.

(6) Effect of training on serum enzyme increases

Exercise A was repeated at weekly intervals for 4-5 weeks. On every occasion blood samples were taken as before and enzyme activities measured. In all 2 animals were studied in Exercise A. Typical results obtained in response to this regime expressed as a percentage of the increase on the first exposure to the exercise are shown in Fig. 2.3. The magnitude of the increase in the concentration of serum enzymes for each animal diminished as the experiment progressed. Four animals were subjected to Exercise B as above and a similar effect was noted on the serum enzyme concentration in each case.

(7) Effect of horse type on serum enzyme increases after exercise

Horses of different types, i.e. thoroughbred and hunter geldings or mares, were used in this study. No significant or

FIG. 2.2. EFFECT OF EXERCISE ON SERUM LDH, CK, ALD AND GOT LEVELS.

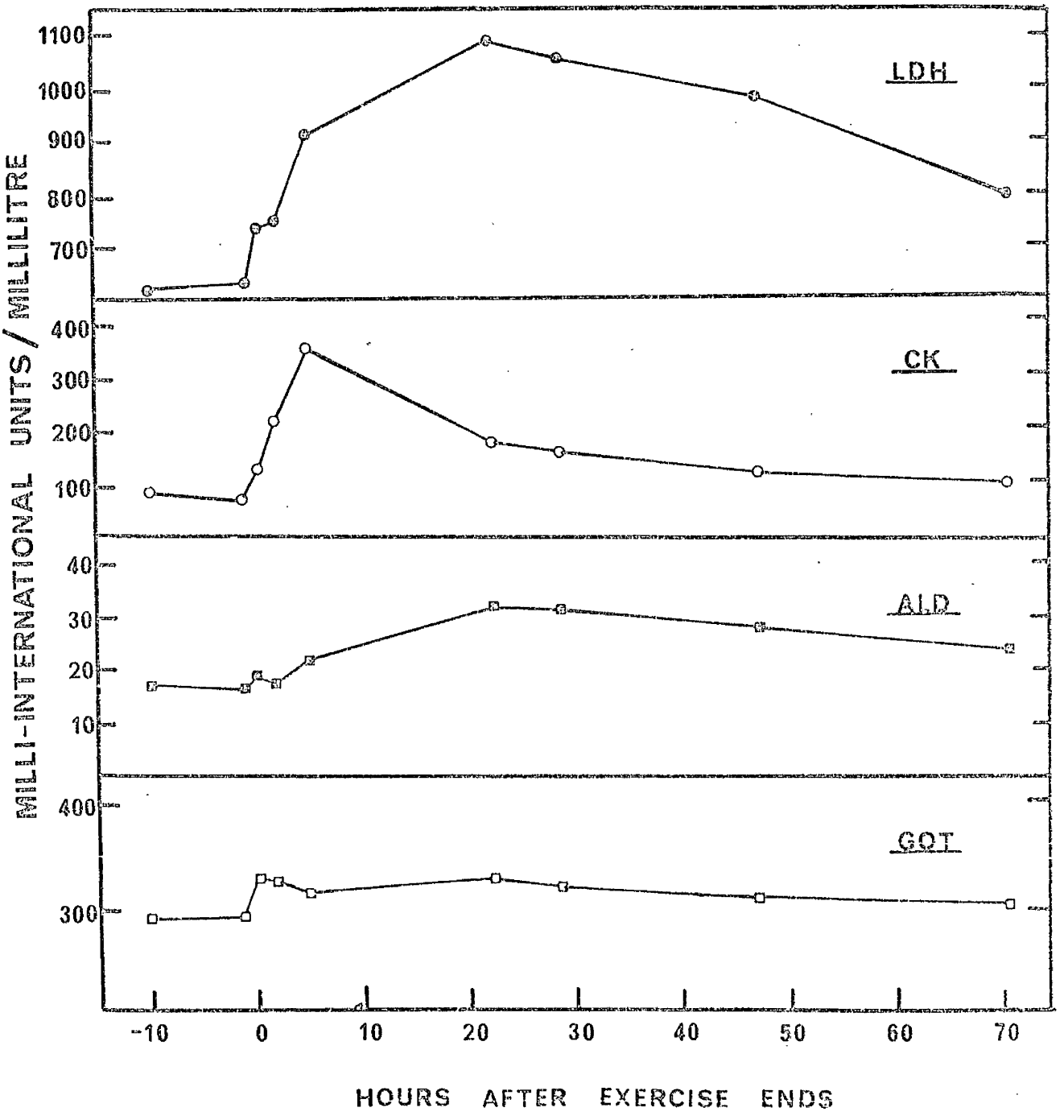
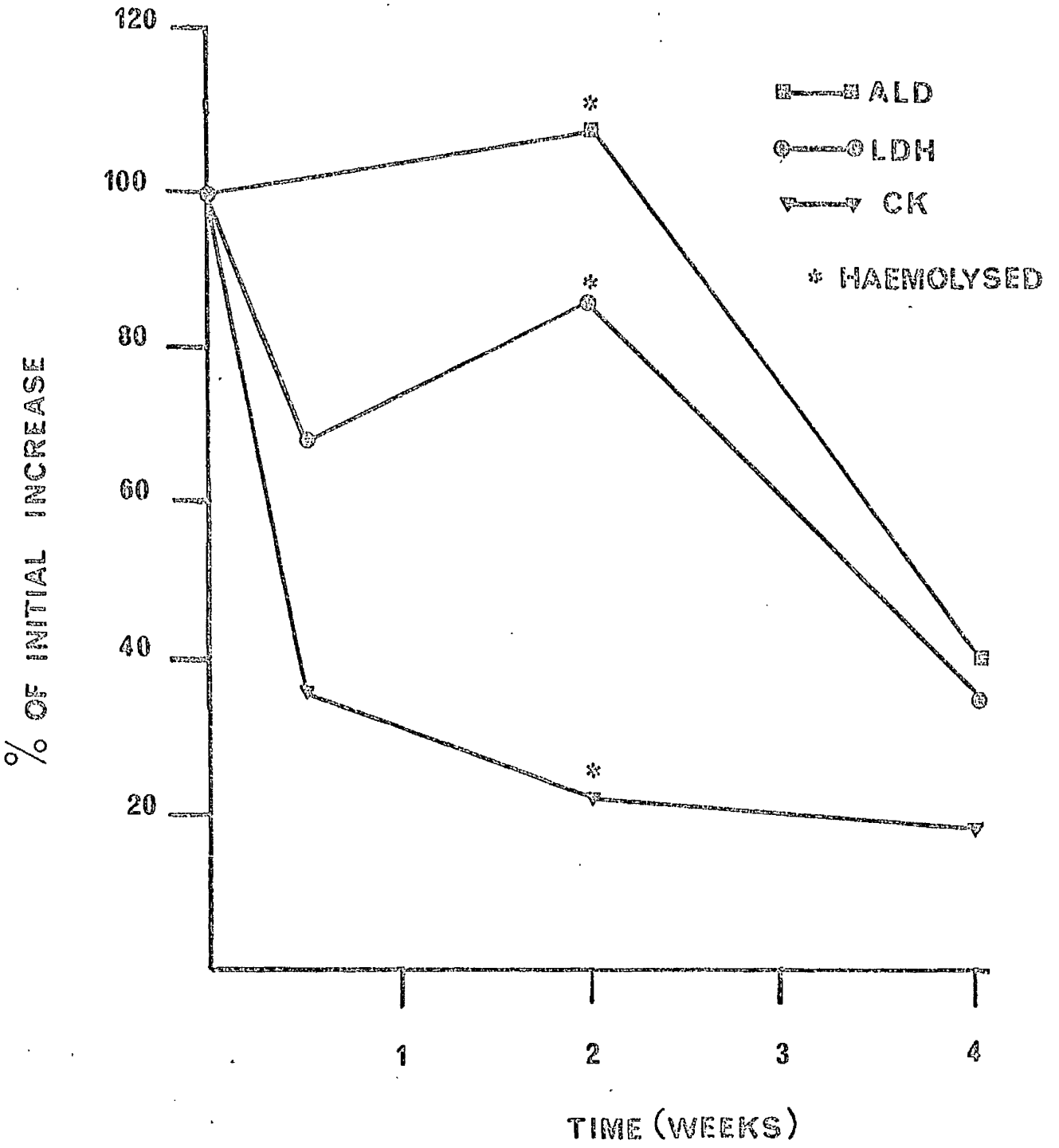


FIG. 2.3. EFFECT OF TRAINING ON MAGNITUDE OF INCREASE IN SERUM ENZYMES



consistent differences between these types were observed in the increases in serum enzyme activities resulting from exercise. Table 2.6 shows the effect of Exercise B on the serum levels of 6 horses - 3 hunters and 3 thoroughbreds. There appears to be no correlation between the magnitude of the increases and the type or sex of the animal.

(8) Effect of distance and pace on serum enzyme increases

The effect of varying the degree of exercise on the magnitude of the enzyme increases has been studied. Two horses, both hunter geldings, were subjected to Exercise A followed 3-5 weeks later by Exercise B. When the enzymatic responses to each distance are compared (Table 2.7) it appears that the increases are directly dependent on the amount of exercise. Two more horses, however, were subjected first to Exercise B then to Exercise A with only one week between. In this instance, the enzymatic response was similar for each exercise probably as a result of a training effect.

A comparison was also made of the response of 4 horses to Exercise B and Exercise C, i.e. the effects of galloping and cantering were compared. Table 2.8 shows the effect of pace on the serum enzyme levels of each horse. The response to Exercise C, which consists of trotting and cantering only, was just as great if not greater than that to Exercise B which included a 4 Km gallop.

TABLE 2.6. EFFECT OF HORSE TYPE ON SERUM ENZYME INCREASES AFTER EXERCISE

Enzyme	Increases after 10 Km Exercise (Exercise B)											
	Horse 13		Horse 14		Horse 3		Horse 2		Horse 12		Horse 6	
	mIU/ml	% of pre-exercise level	mIU/ml	% of pre-exercise level	mIU/ml	% of pre-exercise level	mIU/ml	% of pre-exercise level	mIU/ml	% of pre-exercise level	mIU/ml	% of pre-exercise level
LDH	191	27.6	326	45.5	111	11.9	251	42.3	266	39.0	297	58.3
CK	58	41.8	60	62.4	49	26.9	66	41.5	20	11.3	67	81.6
ALD	11.1	89.5	5.9	54.4	5.7	34.0	19.5	131.6	18.5	113.6	9.0	83.6
GOT	7	3.0	28	11.0	16	6.0	27	10.8	13	4.6	14	6.9

Horse 13 Hunter gelding
Horse 14 " "
Horse 3 Hunter mare
Horse 2 Thoroughbred gelding
Horse 12 " "
Horse 6 Thoroughbred mare

TABLE 2.7. EFFECT OF DISTANCE ON SERUM ENZYME INCREASES

Horse	Enzyme	Increase with Exercise A		Increase with Exercise B	
		mIU/ml	% of pre-exercise level	mIU/ml	% of pre-exercise level
1	GOT	38	13.1	20	6.5
	LDH	512	89.2	174	29.8
	ALD	8.6	119.3	6.0	77.0
	CK	283	410.0	72	111.3
13	GOT	46	16.4	7	3.0
	LDH	500	56.1	191	27.6
	ALD	7.5	53.6	11.1	89.5
	CK	157	116.0	58	41.8
14	GOT	22	8.3	28	11.0
	LDH	237	26.2	326	45.5
	ALD	5.2	40.0	5.9	54.4
	CK	58	56.4	60	62.4
15	GOT	54	13.1	68	11.7
	LDH	179	51.8	121	28.0
	ALD	5.8	46.4	6.6	56.0
	CK	319	269.0	387	407.0

TABLE 2.8. EFFECT OF PACE ON ENZYME INCREASES

Horse	Enzyme	Increase with Exercise B		Increase with Exercise C	
		mIU/ml	% of pre-exercise level	mIU/ml	% of pre-exercise level
3	GOT	16	6.0	41	11.4
	LDH	111	11.9	304	47.3
	ALD	5.7	34.0	12.7	127.0
	CK	49	26.9	380	206.0
6	GOT	14	6.9	58	16.6
	LDH	297	58.3	662	72.5
	ALD	9.0	83.6	10.7	57.0
	CK	67	81.6	347	258.0
12	GOT	13	4.6	15	5.3
	LDH	266	39.0	26	4.8
	ALD	18.5	113.6	2.2	26.9
	CK	20	11.3	19	21.0
14	GOT	28	11.0	28	10.5
	LDH	326	45.5	261	28.9
	ALD	5.9	54.4	7.0	53.8
	CK	60	62.4	143	139.0

DISCUSSION

Although enzyme measurements in serum or plasma have long been used as diagnostic tools in clinical medicine, no rules about the use of serum or plasma exist. With a few exceptions, the choice is usually left to the investigator.

Some workers have found the LDH and GOT activity of serum to be higher than that of plasma (Solbach, Engelhardt and Merten, 1962; Friedel and Mattenheimer, 1970) while others have found no significant differences between serum and plasma (Bessman, Hawkins and Mirick, 1960; Hess, 1963). Friedel and Mattenheimer (1970) have produced strong evidence to suggest that the increased levels in serum are due to the release of enzymes from platelets during the clotting process. Using the method described above for the preparation of serum, most of the cellular elements of the blood, including platelets, were removed prior to clotting and could not, therefore, contribute to serum LDH etc. Such differences in the methods of serum preparation could account for the variable results.

Another factor possibly influencing the relative enzyme activities of serum and plasma is the type of anti-coagulant used in plasma preparation. The increased serum enzyme activities found by Solbach et al (1962) were probably

attributable partly to enzyme release from platelets and partly to their use of a 3.8% sodium citrate solution as anticoagulant. According to Friedel and Mattenheimer (1970), this solution is hypertonic and results in a shift of fluid from erythrocytes into plasma, thus diluting the plasma. The observed increase in serum enzyme activity may, therefore, have been due to decreased activity in plasma rather than increased levels in serum. In the present study, heparin was used as the anticoagulant and the results (Table 2.3) show no difference in the GOT, LDH, ALD and CK activity of serum and plasma prepared from the same blood sample. It would appear that heparin has no effect on the activities of horse GOT, LDH, ALD or CK. It is of interest to note that previous investigations on human blood showed that heparin inhibited human CK (King, 1965). In agreement with our findings heparin had no effect on human GOT, LDH and ALD. At the moment no satisfactory explanation can be given for the different effect of heparin on horse and human CK.

The normal ranges obtained in the horses studied for serum GOT, LDH and CK (Table 2.4a) are higher than those reported previously in the same species (Table 2.4b). This may be a result of different methods of assay of the enzymes or of different conditions of maintaining the animals.

Cardinet et al (1967) and Gerber (1965a) determined GOT

by the method of Reitman and Frankel (1957) as modified by Sigma (1964), LDH and ALD by Boehringer Biochemica Test Kit methods and CK by the method of Tanzer and Gilvarg (1959). It should be noted that in these methods the temperature used is 25°C as opposed to 37°C in the present studies and moreover the conditions employed for GOT and LDH are not optimal for the horse enzymes (Section I). The most striking difference is in the normal values given for CK, the present studies giving normal values 100 times greater than those obtained by Cardinet et al (1967). The method of Tanzer and Gilvarg (1959) is based on the reverse reaction of CK which is less sensitive than the forward reaction. In addition, glutathione is not added in their method. As seen in Fig. 1.32 glutathione reactivates CK and is now used routinely in CK assays since its addition makes the assay system many times more sensitive.

The normal range of serum GOT, LDH, ALD and CK is dependent on the physiological state of an animal. Cornelius, Burnham and Hill (1963) and Cardinet, Fowler and Tyler (1963) both observed that normal serum GOT levels in racehorses undergoing heavy training were higher than in animals not in training or only undergoing moderate daily exercise. It is for this reason that a strict control must be made on the daily exercise of each horse and when quoting normal ranges the nature of the exercise regime should be stated.

It has been shown that in man (Schlang, 1961; Halonen and Konttinen, 1962; Griffiths, 1966) increased serum levels of GOT, LDH, ALD and CK have resulted from severe muscular exercise. Similarly in dogs (Bedrack, 1965), elevated levels of LDH and, in rats (Highman and Altland, 1963; Garbus, Highman and Altland, 1964), elevated levels of LDH, GOT and ALD have been observed after exercise. Moreover, training, in the case of the human and the rat, was found to reduce or eliminate completely serum enzyme increases due to exercise.

Earlier studies carried out on the horse showed increased levels of serum GOT following strenuous swimming exercises (Cornelius et al, 1963). In later studies in the same species, however, Cardinet et al (1967) were unable to confirm these results and observed no significant change in serum GOT following strenuous exercise. However, following the same exercise routine, these latter workers did observe in one animal a dramatic increase in serum CK which was reduced by training.

In the present studies, using a number of horses maintained under strictly defined conditions, the effect of exercise on a greater number of serum enzymes has been examined. After exercise, elevations were observed in serum LDH, ALD and CK levels but no significant change in serum GOT was noted. (Fig. 2.2 and Table 2.6). These increases were seen to decrease as the

animal became trained to the exercise (Fig. 2.3).

The mechanism by which cellular enzymes are released into the bloodstream is still obscure. There has been considerable speculation in the past on whether this phenomenon is due to a temporary change in the permeability of the cell membrane or to damage resulting in destruction of the cell with the release of its contents into the bloodstream. In fact, both mechanisms appear to be operating and the severity of the membrane changes may be indicated by the duration of serum enzyme increases.

In the case of myocardial infarction which results in destruction of cardiac muscle cells, serum levels of GOT, LDH, ALD and CK in man become greatly elevated and remain so for periods ranging from 10 days for LDH to 2-3 days for CK (La Due, Wroblewski and Karmen, 1954; Volk, Losner, Aronson and Lew, 1956; Dreyfus, Schapira, Scebat, Resnais and Lenegre, 1960; Hamolsky and Kaplan, 1961). Similarly, during a microscopic examination of tissue biopsies taken from rats after 16 hours exercise, Garbus et al (1964) observed fatty changes and necrosis in the thigh muscles of some untrained rats. In this case also, serum LDH and ALD levels remained elevated for several days. It appears, therefore, that where there is death of cells, a prolonged increase in serum enzyme activities is noted.

There is, however, evidence that even damage which does not result in cell death can cause the release of enzymes into the

serum and that the resulting increases may persist for some time. Several of the tissue samples examined by Garbus et al (1964) showed no visible signs of necrotic muscle fibres by routine microscopy and yet elevated serum enzyme levels were found in the animals from which these biopsies were taken.

In addition, Halonen and Konttinen (1962) found transient increases in LDH and ALD following 2 hours marching by trained soldiers. In this case, the levels had almost returned to normal within 2 hours of completion of the exercise and the increases were suggested to be the result of some temporary change in the permeability of muscle cell membranes.

Such transient rises may be partly due to the concentration of plasma as a result of intravascular water loss to extravascular compartments during exercise. Cardinet, Littrell, and Schalm (1964) observed an increase in plasma protein concentration of 18% in the horse after 2 hours exercise. By this reckoning, a slight increase in the activity of serum enzymes would also be expected. Assuming a similar situation exists in the human, this would not, however, be sufficient to account for the large transient increases observed in serum LDH and ALD by Halonen and Konttinen (1962). These transient increases and the prolonged increases observed in disease states possibly represent extremes of the same phenomenon i.e. alterations in the cell

membrane ranging from a temporary change resulting in increased permeability to the complete breakdown of the membrane structure with the death of the cell.

Since the enzyme levels in this study vary in a manner resembling that seen following myocardial infarction except that the increases do not persist quite as long, it could be suggested that the exercise has caused some necrosis of muscle cells. In the case of an untrained though healthy horse subjected to moderately severe exercise for 30-60 minutes, however, it seems unlikely that such exercise should cause muscle cell death. An alteration in cell membrane permeability seems more probable. The present findings suggest cell damage intermediate in severity between that found in pathological conditions such as myocardial infarction and that indicated by the transient serum enzyme increases found by Halonen and Konttinen (1962).

Whatever the mechanism, it is interesting to note the different times at which peak levels of CK and LDH appear. Peak CK levels are found around 5 hours after exercise in contrast to LDH and ALD which peak around 24 hours. This may be due to faster release of CK from cells or to a faster elimination rate. Relatively little is known about the half-lives of serum enzymes in domestic animals and, of the information available, there is considerable variation in results from different workers. This

is probably due to the fact that some workers have calculated the half-lives from the clearance of radioactively-labelled enzymes injected into animals while others have used the time required for the serum levels to return to normal after injury. In this latter method, the half-life may be overestimated since it is assumed that there is no further release of enzymes into the bloodstream after injury. The only information available for the horse appears to be the half-life of equine CK which is quoted as 108 minutes (Cardinet et al, 1967). These workers did also, however, observe that equine GOT was cleared much more slowly from the bloodstream although they did not actually obtain a value for the half-life.

The molecular weight of CK from rabbit muscle (Noda, Kuby and Lardy, 1954) is 81,000 - in other words, it is a smaller molecule than LDH or ALD which have molecular weights of 135,000-147,000 and 180,000 respectively (Dixon and Webb, 1958). Since the molecular weights of enzymes from widely different vertebrate sources are similar, the horse enzymes will not differ greatly from these. This might suggest that, because of its size, CK passes through the cell membrane more easily than larger molecules such as LDH. In experiments with perfused rat liver, Schmidt and Schmidt (1967) showed that cytoplasmic enzymes leaked from the liver in inverse proportion to their molecular

weight. This might be expected if the leakage was due to passive diffusion since small molecules diffuse more rapidly than larger ones. Passive diffusion would also result in enzymes leaking out in proportion to their intracellular concentration.

Although the sequence of serum enzyme increases following exercise appears to indicate passive diffusion, the phenomenon cannot be explained by this alone. GOT, which, with a molecular weight of 116,000 (Sizer and Jenkins, 1962) is smaller than ALD and is present in high intracellular concentrations, is not released to any great extent during exercise. This is surprising since GOT is mainly cytoplasmic and since all 4 enzymes are found in elevated serum levels in equine paralytic myoglobinuria (Gerber, 1965a). This latter disorder, however, probably results in necrosis of the cell with rupture of the cellular membrane. In this case, all of the cell's cytoplasmic and eventually particle-bound enzymes would be released into the extracellular fluid. The fact that GOT is not released in the present studies supports the idea that the release is not due to cell breakdown but to a selective change in membrane permeability. The earlier peak of CK activity in horse serum following exercise may be due, therefore, to both the smaller size of CK and to its faster elimination rate from the circulation.

Since the mechanism of release of cellular enzymes into

the bloodstream is still unknown, one can only speculate on the manner in which training reduces the enzyme increases resulting from exercise. It seems likely that training either makes the cell membrane less susceptible to the stimulus which causes the permeability change or it reduces the stimulus. Garbus et al (1964) suggested that susceptible portions of the cell membrane, damaged during training, might be replaced by less susceptible elements. Hypoxia has been shown to stimulate the release of cellular enzymes from perfused rat tissues (Zierler, 1958; Schmidt, Schmidt, Herfarth, Optiz and Vogell, 1966). If the enzymatic effects of exercise were due to tissue hypoxia, training could reduce this effect by increasing the capacity of the cardiovascular system to supply oxygen to the tissues. In fact, haematocrit and blood volume have been shown to increase in racehorses during training (Persson, 1968). Whether or not hypoxia is of importance in contributing to enzyme increases during exercise in the horse will be discussed later (Section IV).

In the present studies, a slight decrease is observed in the pre-exercise serum enzyme levels over the 4-5 week training period i.e. as the training progressed the normal resting levels decreased. This might indicate that the training process reduced the susceptibility of cell membranes to the normal "wear and tear" process which accounts for the

enzyme activity normally present in serum.

The enzymatic response in the horse seems to depend more on the duration of the exercise than on the distance or pace. Exercise C, because it is composed of trotting and cantering only, takes longer to complete than Exercise B which contains a gallop. The results obtained above show that the enzymatic response following Exercise C is, if anything, greater than that following Exercise B, which would suggest that the length of time for which the animal is exposed to some stimulus arising from exercise may be critical rather than the magnitude of the stimulus. The nature of such a stimulus will be considered later.

The fact that no difference was observed in the response of horses of different types to the same exercise probably reflects the training method rather than genetic factors. In this study, all of the horses were exposed to the same daily routine and diet. Under normal circumstances hunters and thoroughbreds would be trained by different methods to correspond with the type of event in which the animal would participate.

In conclusion, therefore, the present studies show that serum levels of LDH, ALD and CK increase following exercise in the horse. Results for GOT are inconclusive. The magnitude of the increases is dependent on the state of training of the animal

and indicates that the measure of the increase could be the basis of a test for assessing fitness in the horse. Since CK is the most specific of these enzymes, this would appear to be the enzyme of choice in such studies.

SECTION III

Studies on Isoenzyme Patterns of Horse Serum and Tissues

INTRODUCTION

In this section, the LDH and CK isoenzyme patterns of various horse tissues are described. The isoenzyme patterns in serum before and after exercise are also examined to determine the tissue or tissues of origin on the increased amount of LDH and CK in serum resulting from exercise.

CK has been shown to exist in multiple molecular forms by electrophoretic separation on a variety of media. Electrophoresis of human tissue extracts and pathological sera on agar gel (Deul and van Breeman, 1964; Van der Veen and Willebrands, 1966) and cellulose acetate (Rosalki, 1965; Trainer and Gruenig, 1968) demonstrated the existence of three CK isoenzymes. One of these forms, however, was separated into three further bands of activity by electrophoresis on starch gel (Sjövall and Voigt, 1964) and polyacrylamide (Sjövall and Jergil, 1966).

Numerous separations of LDH isoenzymes in tissues and sera from a great variety of species have been reported previously. Five isoenzymes of LDH have been demonstrated following separation on starch gel (Wroblewski, Ross and Gregory, 1960), agar gel (Van der Helm, 1962), paper (Raabo, 1963) and cellulose acetate (Barnett, 1964). Polyacrylamide has also been used for this purpose by Matson (1963), Schrauwen (1966) and Dietz and Lubrano (1967). In

addition to the five LDH isoenzymes normally seen, other forms of this enzyme have been reported in the testis and spermatozoa of some mammals (Clausen and Øvlisen, 1965; Goldberg, 1963; Valenta, Hyldgaard-Jensen and Moustgaard, 1967) and birds (Zinkman, Blanco and Kupchyk, 1963).

Previous separations of LDH isoenzymes in the horse have been carried out on agar or starch gel (Gerber, 1966; Coffman, Mussman and Cawley, 1969) with variable results. Gerber (1966) found six LDH isoenzymes in horse liver in contrast to Coffman et al (1969) who found five. In addition Gerber (1966) found very little LDH_5 in horse skeletal muscle - a surprising finding in the light of the high LDH_5 content of skeletal muscle reported in other species (Wroblewski and Gregory, 1961; Hyldgaard-Jensen, 1971).

In this work, the separation of LDH and CK isoenzymes in horse serum and tissue extracts is carried out in polyacrylamide since the resolving power of this material is high and the medium clear allowing easy quantitation of bands.

MATERIALS AND METHODS

Materials

The following is a list of materials used in this section together with their sources. All reagents are "Analar" grade from BDH Chemicals Ltd., unless otherwise stated.

Chemicals

Adenosine 5'-diphosphate (sodium salt) from Sigma.

Adenosine 5'-monophosphoric acid.

Ammonium persulphate.

Bromophenol Blue.

'Cyanogum'.

D(+) Glucose.

Glutathione, reduced form (Sigma).

Horse albumin (Cohn fraction V) from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Iodoacetic acid.

Magnesium acetate.

Magnesium chloride.

β-Nicotinamide-adenine dinucleotide (NAD^+) from Boehringer Corp.

β-Nicotinamide-adenine dinucleotide phosphate ($NADP^+$), sodium salt (Sigma).

Nitro Blue Tetrazolium.

Phenazine methosulphate.

Phosphocreatine disodium salt hydrate (Sigma).

'Repelcote' (Hopkin & Williams Ltd., Chadwell Heath, Essex, England).

Sodium chloride.

Sodium lactate, 70% solution.

N,N,N',N'-tetramethyl-1,2-diminoethane (TEMED) from Koch-Light Laboratories Ltd.

Trichloracetic acid.

Enzymes

Glucose-6-phosphate dehydrogenase, sulphate free (Sigma).

Hexokinase, sulphate free (Sigma).

Buffers

Glycine.

Potassium dihydrogen orthophosphate.

Triethanolamine hydrochloride (Sigma).

Tris-(hydroxymethyl)-methylamine (TRIS), Aristar.

Methods

Preparation of tissue extracts

Tissues were taken from 2 horses within 2 hours of death (one horse having been shot and the other having died naturally of grass-sickness), and 10% homogenates prepared. The tissues were washed to remove as much blood as possible (perfusion is not advisable because of possible loss of enzymes) and 2 gram of each chopped up finely with scissors and suspended in 19 ml. ice cold 0.25M-sucrose. After homogenising in a mixer-emulsifier (Silverson) for 2-3 minutes with frequent cooling, the suspension was cleared by centrifuging at 20,000 g. for 20 minutes at 5°C in a Beckman L2-65B ultracentrifuge. Unless assays were carried out immediately, the supernatants were stored at -15°C for periods not exceeding 4 days. Erythrocyte extracts were prepared by washing red cells 3 times with 0.25M-isotonic sucrose, suspending in 6 volumes of distilled water and centrifuging to remove cell debris. Contamination of erythrocyte suspensions and lysates by other blood cells was considered insignificant by microscopic examination.

The haemoglobin concentration of haemolysates was determined by the cyanmethaemoglobin method. Enzyme assays were carried out on the tissue extracts by the methods described in Section I. In the estimation of CK, each assay had to be accompanied by a blank because of high blank activity. When

necessary, the extracts were diluted with the appropriate buffer containing 1% horse albumin.

Electrophoretic separation of LDH isoenzymes

Dietz and Lubrano (1967) have described a method which employs a single gel for separation and uses a concentrated sucrose solution to layer the sample on top of the separating gel, thus avoiding the use of sample and spacer gels (Davis, 1964). This method formed the basis of that described below with several major modifications.

Reagents

Gel Buffer (0.377M-Tris, HCl buffer, pH 8.9):- 45.75 g. Tris were dissolved in 600 ml. distilled water and the pH brought to 8.9 with 1M-HCl. The volume was made up to 1 litre with distilled water and the pH checked. (Stored at $4^{o}C$).

15% acrylamide solution:- 15 g. 'Cyanogum' were dissolved in gel buffer to give a final volume of 100 ml. (Stored in a brown bottle at $4^{o}C$).

Electrophoresis buffer (0.01M-Tris, 0.05M-glycine, pH 8.5):- 24.1 g. Tris and 76.1 g. glycine were dissolved in distilled water to make 1 litre. For anode buffer this solution was diluted 1:10 with water and the pH checked. For cathode buffer, 100 ml. of the solution was diluted with 600 ml. water and 1.86 g. iodoacetic acid added. The pH was adjusted to

8.5 with 1M-NaOH and the volume made up to 1 litre. 1 drop 1% bromophenol blue/500 ml. cathode buffer was added. (Stored at 4°C).

7% Ammonium persulphate solution:- 0.7 g. ammonium persulphate were dissolved in water to give 10 ml. solution. (Prepared fresh weekly and stored at 4°C).

1% Bromophenol blue solution:- 0.1 g. bromophenol blue were dissolved in water to give 10 ml. solution. (Stored at 4°C).

Sucrose solution:- 40 g. sucrose were dissolved in distilled water to give 100 ml. solution. (Stored at 4°C).

Staining solution:- The modified van der Helm (1961) staining solution adopted by Dietz and Lubrano (1967) was used here.

Apparatus

A disc electrophoresis apparatus of the type described by Ornstein (1964) and Davis (1964) capable of holding 12 tubes was used (Fig. 3.1). Tubes 10 cm. long and with an i.d. of 7 mm. were prepared by rinsing first in distilled water, then in Repelcote and drying in a coolish oven (40-50°C).

Method

5% gels were prepared by mixing -

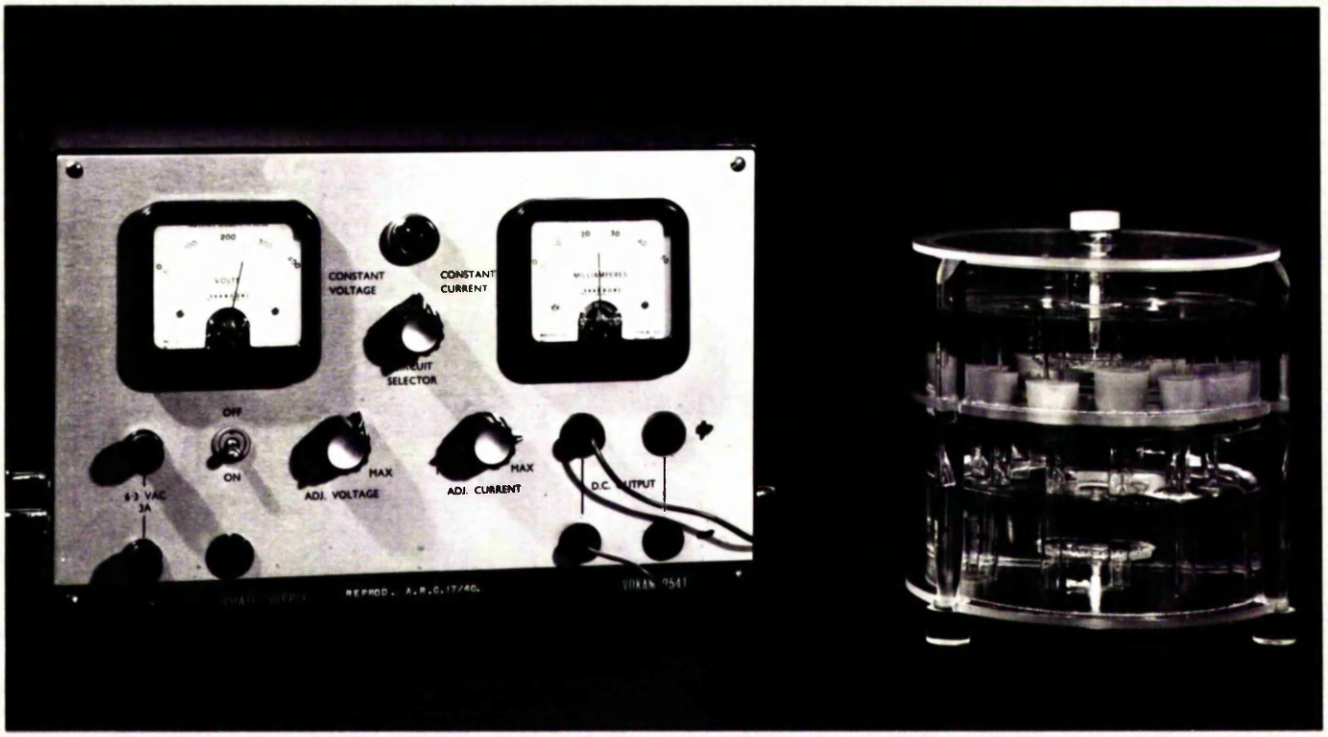
10.0 ml. 15% acrylamide

19.64 ml. gel buffer

0.06 ml. TEMED

0.30 ml. ammonium persulphate.

FIG. 3.1. DISC ELECTROPHORESIS APPARATUS



2.0 ml. of this solution was quickly pipetted into each tube (gel length approximately 5.3 cm.) and carefully overlaid with water to a depth of 3-5 mm. After polymerisation (about 20-30 minutes) the gels were marked 3.5 cm. below the surface and transferred to a cold room at 4°C. The water layer was replaced by cold cathode buffer (changed twice) and the cathode chamber filled with this buffer. After filling the lower chamber with anode buffer, electrophoresis was carried out at 2.5 mA/tube for 30 minutes to flush iodoacetate into the gels.

Samples were prepared by diluting tissue extracts to give activities around 500 mI.U/ml. and adding to an equal volume of 40% sucrose. Sera were diluted 1:1 with sucrose. 50 µl. of each sample was injected with a syringe directly on to the top surface of the gel and electrophoresis carried out at 1 mA/tube for 15 minutes or until the sample has entered the gel. (The sample is visible as a bluish band due to the binding of bromophenol blue to albumin in the serum). The current was then increased to 4 mA/tube and electrophoresis carried out until the albumin band (stained with bromophenol blue) reached the 3.5 cm. mark (2-3 hours). Free bromophenol blue will migrate as a blue band ahead of this albumin dye complex. The gels were rimmed by the method of Reisfeld, Lewis and Williams (1962), transferred to small tubes (i.d. 10 mm.) containing 3 ml. of staining solution, covered with Parafilm and incubated at 37°C for 30-45 minutes -

staining beyond this time favours the low activity bands. When the gels were adequately stained, they were washed with tap water and placed in 5% trichloroacetic acid. Normally 1 blank was sufficient for each batch of gels. In this case, lactate was omitted from the staining solution.

The isoenzyme bands were quantitated using a densitometer (Kipp & Zonen, Delft, Holland).

Electrophoretic separation of CK isoenzymes

CK isoenzymes were separated on polyacrylamide by the method described below which uses the technique of Sjövall and Jergil (1966) i.e. the electrophoretic inclusion of HK and GPDH in the gel.

Reagents

Electrophoresis buffer (0.01M-Tris, 0.05M-glycine, pH 8.5):- Anode buffer was prepared as for LDH anode buffer. To prepare cathode buffer, 100 Units hexokinase, 50 Units glucose-6-phosphate dehydrogenase and 1 drop 1% bromophenol blue were added to 500 ml. of buffer, just before use.

Staining solution:- The composition of the staining solution was based on the optimal conditions for horse CK determined in Section I and it was made up as follows: 0.606 g. creatine phosphate, 48.5 mg. ADP, 0.2833 g. $NADP^+$, 0.3603 g. glucose, 0.3642 g. AMP and 0.2145 g. magnesium acetate were dissolved

in 60 ml. distilled water. 10 ml. 1.0M-triethanolamine buffer were added, the pH adjusted to 7.0 with 1M-NaOH and the volume made up to 100 ml. with distilled water. The solution was divided into 20 ml. batches and stored at -15°C. Immediately before use, 2 ml. nitro blue tetrazolium (5 mg/ml.) and 0.60 ml. phenazine methosulphate (1 mg/ml.) were added to each batch.

Each 20 ml. volume was sufficient for 6 gels.

Method

The procedure was the same as for LDH isoenzymes with the electrophoresis buffers replaced by those for CK. Samples were prepared by diluting serum 1:1 with 40% sucrose containing glutathione at a concentration of 1 mg/ml., tissue extracts being first diluted to 2,000-5,000 mI.U/ml. Electrophoresis was carried out at 1 mA/tube for 15 minutes followed by 2.5 mA/tube until the albumin band reached the 3.5 cm. mark.

The gels were incubated in staining solution at 37°C for 1 hour, washed and stored in 5% trichloroacetic acid as before. For CK isoenzymes, it was necessary to run a blank for each sample and this was done by omitting phosphocreatine from the staining solution.

RESULTS

(1) Distribution of GOT, LDH, ALD and CK in some horse tissues

The GOT, LDH, ALD and CK content of brain, cardiac muscle, skeletal muscle, liver, kidney, spleen and erythrocytes was determined for 2 horses and the results are shown in Table 3.1. Muscle samples were obtained from the diaphragm of Horse 17 and the biceps femoris of Horse 18. Both kidney samples were taken from the cortex and both cardiac muscle samples from the ventricles.

GOT is seen to be present in highest amounts in skeletal muscle, cardiac muscle and liver with little activity in erythrocytes relative to the other tissues. LDH is a ubiquitous enzyme present in high concentrations in each of the tissues examined but with heart, muscle and kidney predominating. Skeletal muscle was found to contain 30-50 times as much aldolase as cardiac muscle although the aldolase content of skeletal muscle does seem to depend on the functional activity of muscle. The aldolase activity of erythrocytes is also high relative to the other tissues shown. Creatine kinase is found in appreciable amounts in heart, brain, kidney and spleen with very high concentrations in skeletal muscle. Liver, on the other hand, has a low CK content and the enzyme is virtually absent from erythrocytes.

Table 3.2 shows the tissue/serum activity gradients for

TABLE 3.1. DISTRIBUTION OF GOT, LDH, ALD AND CK IN SOME HORSE TISSUES

Tissue	Horse No.	Units per Gram of Wet Tissue							
		GOT		LDH		ALD		CK	
Brain	17	13.3	(4.9)	77.3	(48.5)	4.2	(0.32)	119.7	(27.6)
	18	13.9		83.6		4.2		134.3	
Cardiac Muscle	17	73.4	(31.7)	324.3	(354.2)	0.2	(0.23)	486.6	(69.4)
	18	57.3		314.9		0.3		752.0	
Skeletal (Diaphragm)	17	118.9	(53.7)	162.2	(154.6)	—	(7.90)	1320.5	(99.2)
Muscle (Biceps Femoris)	18	125.3		476.0		11.2		2753.6	
Liver	17	59.7	(33.4)	46.0	(81.8)	1.1	(0.76)	7.0	(1.48)
	18	39.7		25.7		1.3		7.2	
Kidney	17	10.1	(6.8)	127.0	(121.8)	1.5	(0.57)	46.8	(0.99)
	18	11.2		146.1		2.4		82.3	
Spleen	17	1.7	(3.5)	25.6	(43.1)	0.7	(0.72)	24.4	(1.58)
	18	3.7		74.7		0.8		64.5	
Erythrocytes*	2	2.8		35.3		2.7		0.5	
	14	2.1		32.6		3.5		0.6	
	15	2.2		37.2		1.8		0.2	

* For Erythrocytes, enzyme activity is expressed as units/gram haemoglobin
( ) Figures in brackets are those of Gerber (1964, 1965b)

TABLE 3.2. TISSUE/SERUM ENZYME ACTIVITY GRADIENTS

Tissue	GOT		LDH		ALD		CK	
	Horse	Human[a]	Horse	Pig[b]	Horse	Rat[c]	Horse	Sheep[d]
Brain	49	-	113	203	339	263	1,144	995
Cardiac Muscle	237	7,800	453	435	20	260	5,579	305
Skeletal Muscle	444	5,000	451	4,294	903	1,233	18,351	2,037
Liver	181	7,100	51	178	97	201	64	16
Kidney	39	4,500	194	221	157	130	627	100
Spleen	10	700	71	213	60	80	401	310
Erythrocytes	9	15	50	21	215		4	

Gradients were calculated from units/gram wet tissue divided by units/ml. serum

a from Wroblewski and La Due (1956)
b from Hyldgaard-Jensen (1971)
c from Sibley and Lehninger (1949)
d from Brown and Wagner (1968) and Wagner and Gray (1968).

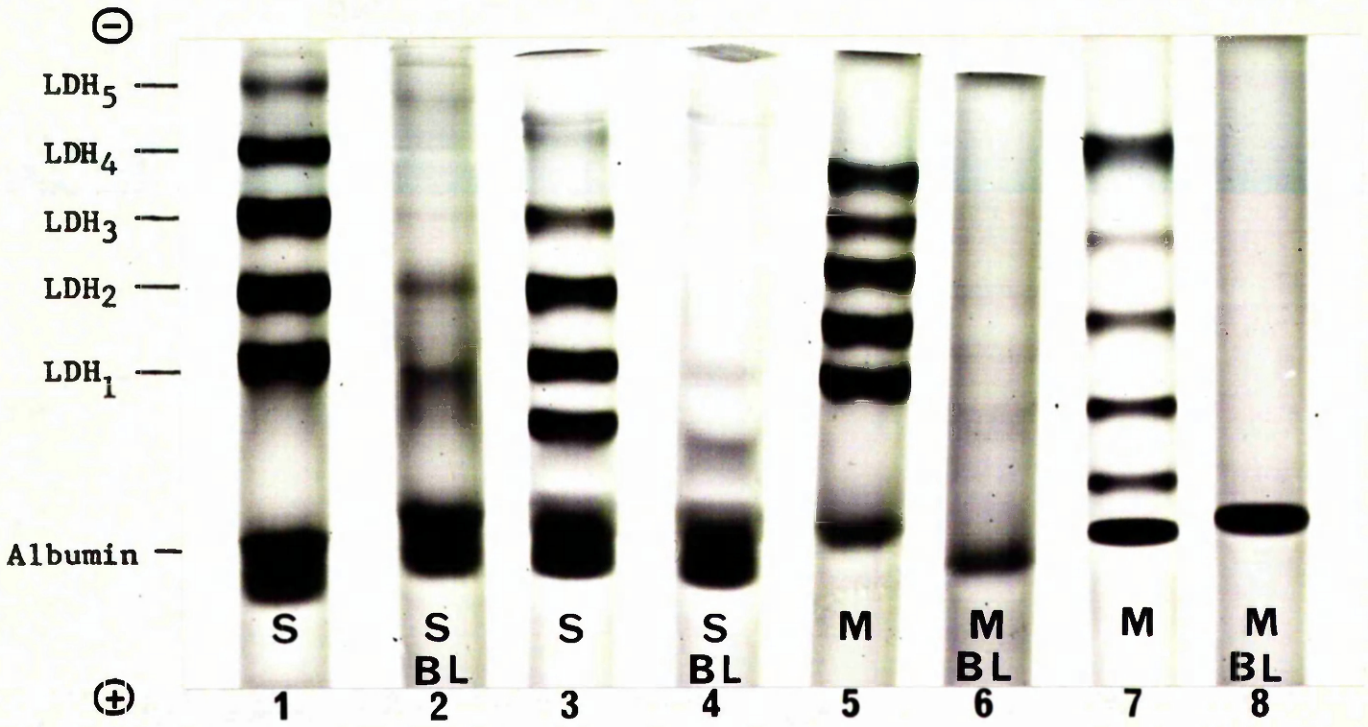
the tissues investigated, together with results taken from the literature on other species.

(2) LDH isoenzyme patterns in some horse tissues

Using the method of Dietz and Lubrano (1967), background staining was observed in these studies. By the electrophoretic inclusion of iodoacetate in the medium, however, the background staining was diminished without affecting the LDH activity. This lends support to the hypothesis that the background staining is due to sulphydryl groups on proteins (Dietz and Lubrano, 1967). Fig. 3.2 illustrates the effect for serum and for muscle extract. Band formation in the blanks was less obvious in the presence of iodoacetate. Table 3.3 illustrates the reproducibility of the method for separation of LDH isoenzymes. The electrophoretic migration distance, i.e. the R_{HA} values for horse LDH isoenzymes relative to horse albumin were calculated (Table 3.4). In numbering the isoenzymes, the recommendation of the Sub Committee on Isoenzymes of the International Union of Biochemistry has been followed, i.e. the isoenzymes are denoted LDH_1, LDH_2, LDH_3, LDH_4 and LDH_5 in order of decreasing electrophoretic mobility.

The distribution of LDH isoenzymes in tissues from one horse is shown in Fig. 3.3. Each of the tissues examined contained 2, 3, 4 or 5 isoenzymes. The composition of tissues from both horses is expressed in Table 3.5 as percentages of the

FIG. 3.2. EFFECT OF IODOACETATE ON BACKGROUND STAINING



S = Serum

S BL = Serum blank

M = Muscle extract

M BL = Muscle blank

Gels (1), (2), (5) and (6) were run in the absence of iodoacetate and gels (3), (4), (7) and (8) in 10 mM iodoacetate.

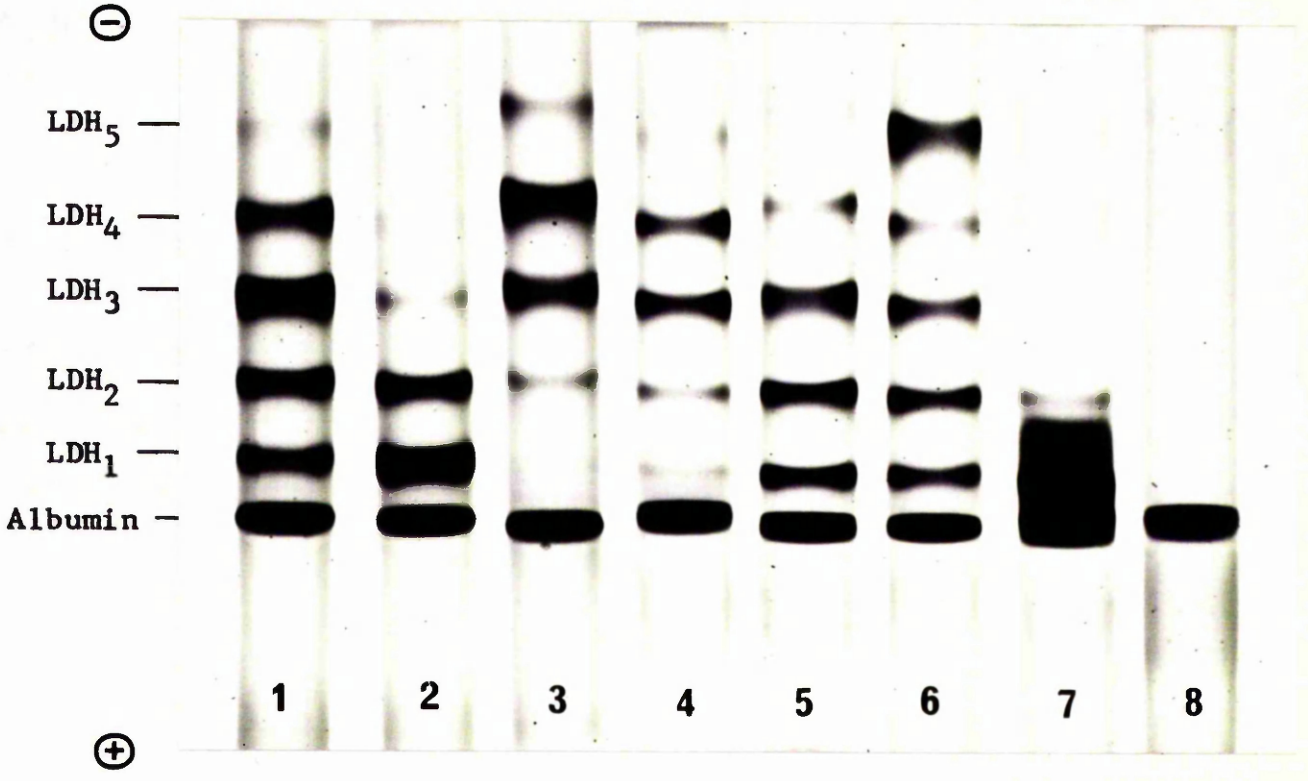
TABLE 3.3. REPRODUCIBILITY OF LDH ISOENZYME METHOD

Sample	LDH_1 %	LDH_2 %	LDH_3 %	LDH_4 %	LDH_5 %
S14-14/6	29.4	24.9	25.1	15.2	5.4
	28.4	23.3	25.3	17.3	5.7
	29.1	24.6	25.1	16.1	5.1
Mean	28.97	24.27	25.17	16.20	5.40
S.D.	0.51	0.85	0.12	1.05	0.30
S.E.	0.30	0.49	0.07	0.61	0.17

TABLE 3.4. ELECTROPHORETIC MIGRATION DISTANCE OF HORSE LACTIC DEHYDROGENASE AND CREATINEKINASE RELATIVE TO THE MIGRATION FRONT OF HORSE ALBUMIN

LDH	R_{HA}	CK	R_{HA}
1	0.73 ± 0.07	I	1.10 ± 0.04
2	0.60 ± 0.06		
3	0.47 ± 0.05	II	0.73 ± 0.06
4	0.32 ± 0.03	III	0.33 ± 0.04
5	0.17 ± 0.01		

FIG. 3.3. LDH ISOENZYME COMPOSITION OF SOME HORSE TISSUES



Gels contain (1) Brain, (2) heart, (3) liver, (4) spleen, (5) kidney, (6) muscle, (7) erythrocyte, (8) muscle blank.

TABLE 3.5. LDH ISOENZYME COMPOSITION OF HORSE TISSUES

Tissue	Horse No.	% of Total Activity				
		LDH_1	LDH_2	LDH_3	LDH_4	LDH_5
Brain	17	13.2	21.5	38.4	23.4	3.5
	18	23.3	24.0	36.3	16.4	0
Cardiac Muscle	17	60.2	34.3	5.5	0	0
	18	65.5	30.1	4.4	0	0
Skeletal Muscle	17	15.0	18.6	17.7	12.1	36.6
	18	6.3	8.2	14.0	3.4	68.1
Liver	17	0	4.2	18.9	47.7	29.2
	18	0	5.2	10.8	37.9	46.1
Kidney	17	24.8	32.9	23.4	12.6	6.3
	18	32.7	29.9	27.1	8.5	1.8
Spleen	17	3.0	11.3	32.3	37.7	15.7
	18	6.6	20.4	34.9	26.2	11.9
Erythrocytes	14	76.8	12.8	4.9	5.5	0
	15	77.3	12.1	3.6	7.0	0

total LDH activity. The only tissue which varied significantly in isoenzyme composition between the two samples examined was skeletal muscle and in this case, samples had been taken from different muscles, i.e. diaphragm and biceps femoris. Diaphragm LDH is composed of 36.6% LDH_5 while this isoenzyme makes up 68.1% of biceps femoris LDH activity.

In horse brain, LDH_3 predominates with equal amounts of LDH_1, LDH_2 and LDH_4. There is very little LDH_5 present in this tissue. The LDH content of cardiac muscle is composed mainly of the anodic isoenzymes with over 60% LDH_1 and 30% LDH_2. No trace of LDH_4 or LDH_5 is found in cardiac muscle. Liver has a similar pattern to skeletal muscle with the cathodic isoenzymes predominating. The LDH_4 content of liver, however, is much higher than that of muscle. LDH_1, LDH_2 and LDH_3 are the isoenzymes present in highest concentrations in kidney while in spleen LDH_3 and LDH_4 are the predominating forms. Quantitation of the bands in erythrocyte extracts was complicated by the fact that horse haemoglobin migrates with LDH_1. Although quite distinct to the naked eye because of its different colour, this band could not be separated from the pigment band by the available densitometer. The results for erythrocytes in Table 3.5 are, therefore, visual estimations.

(3) LDH isoenzyme pattern of horse serum before and after exercise

The normal variation in LDH isoenzyme composition of horse

serum was determined using sera from 8 healthy horses. 4 or 5 isoenzymes are seen in normal serum. The results in Table 3.6 indicate that the most variable isoenzyme is LDH_1, ranging from 15.3% to 42.2% of the total activity.

To study the effects of exercise, serum was taken from 2 horses before and 24 hours after exposure to Exercise B and from 1 horse before and after Exercise C. The LDH isoenzyme composition of these sera is shown in Fig. 3.4 and Table 3.7. This percentage distribution shows that LDH_4 and LDH_5 are increased while the other 3 forms remain constant or are slightly reduced. When the absolute activities of the isoenzymes are expressed, however, it is obvious that all 5 isoenzymes are increased although the increases in LDH_4 and LDH_5 are much more pronounced.

(4) CK isoenzyme patterns in some horse tissues

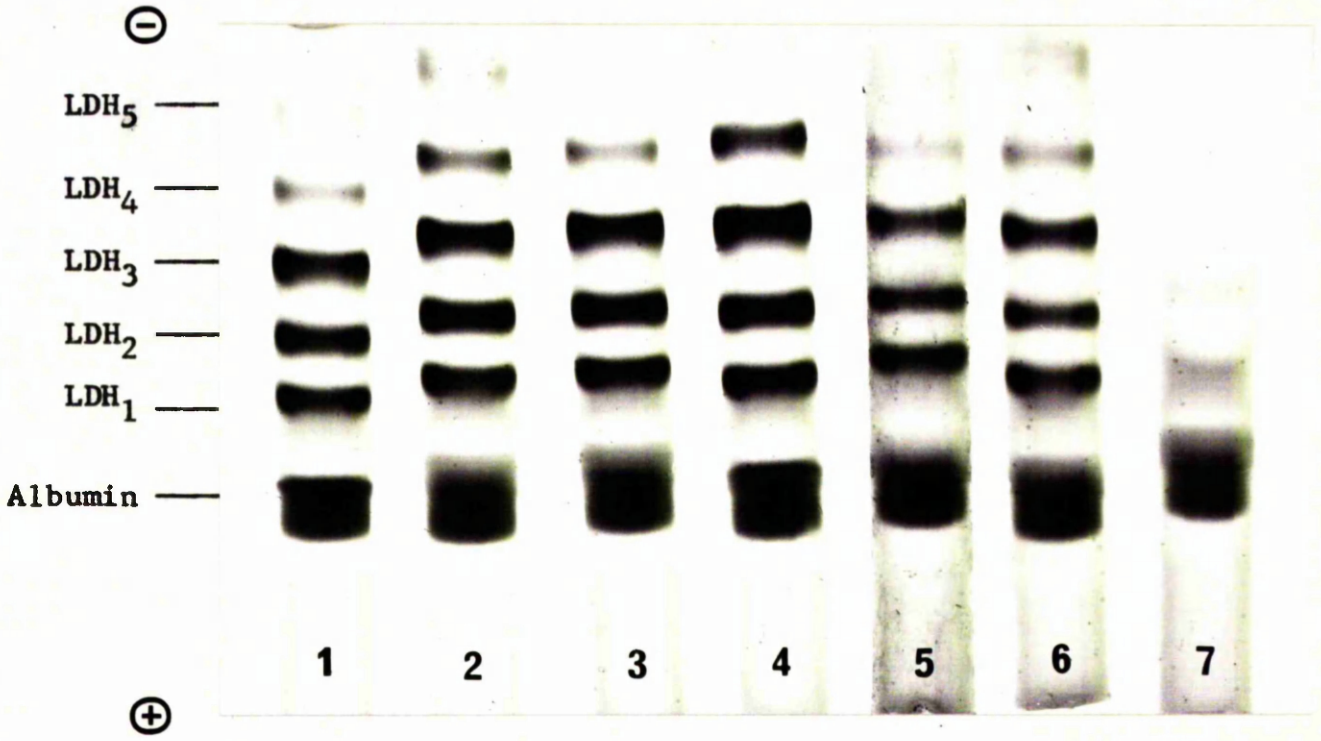
Electrophoresis was carried out using the same tissue extracts as above (with the exception of erythrocytes) and the gels were stained for CK. The results are shown in Fig. 3.5 and 3.6 and Table 3.8. The percentages quoted in Table 3.8 are only approximate because of uneven background staining. CK isoenzymes are numbered I to III in order of decreasing electrophoretic mobility and the R_{HA} values of these isoenzymes relative to horse albumin are quoted in Table 3.4.

The major isoenzyme in brain, kidney, spleen and liver

TABLE 3.6. PERCENTAGE COMPOSITION OF LDH ISOENZYMES OF NORMAL HORSE SERUM (8 ANIMALS)

	% of Total Activity				
	LDH_1	LDH_2	LDH_3	LDH_4	LDH_5
Mean	26.0	23.0	27.0	17.3	6.7
S.D.	$\pm$10.4	$\pm$2.5	$\pm$1.9	$\pm$5.7	$\pm$6.3
Range	15.3-42.4	18.6-26.1	23.4-29.0	11.0-23.9	0-15.3

FIG. 3.4. EFFECT OF EXERCISE ON THE LDH ISOENZYME COMPOSITION OF HORSE SERUM

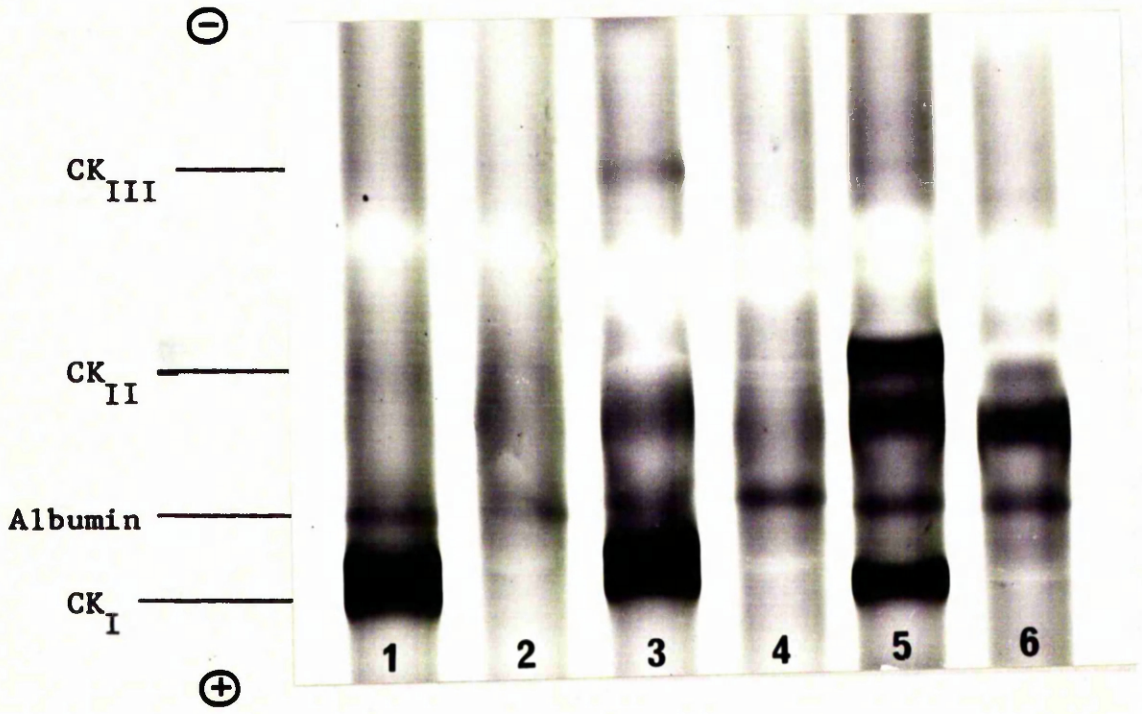


Gels contain serum from (1) Horse 14 before Exercise C
(2) Horse 14 after Exercise C
(3) Horse 14 before Exercise B
(4) Horse 14 after Exercise B
(5) Horse 15 before Exercise B
(6) Horse 15 after Exercise B
(7) Horse 14 blank.

TABLE 3.7. EFFECT OF EXERCISE ON LDH ISOENZYME COMPOSITION OF HORSE SERUM

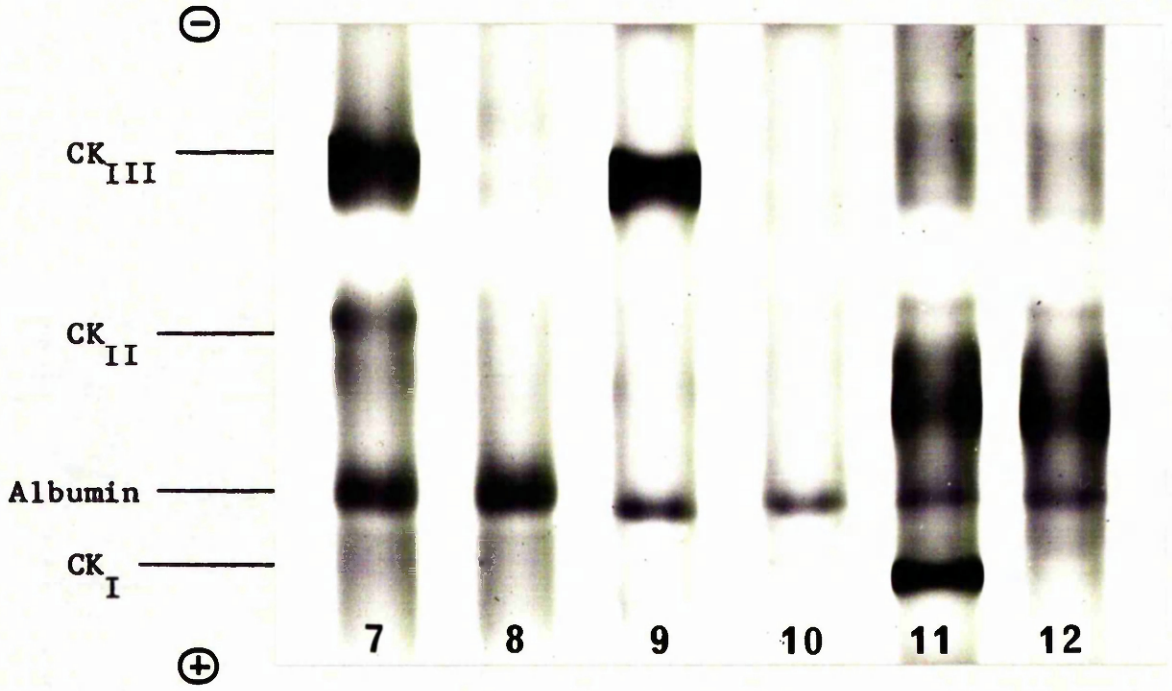
Horse No.	Exercise		Total Serum LDH mIU/ml	LDH_1		LDH_2		LDH_3		LDH_4		LDH_5	
				%	Absolute Activity mIU/ml	%	Absolute Activity mIU/ml	%	Absolute Activity mIU/ml	%	Absolute Activity mIU/ml	%	Absolute Activity mIU/ml
14	C	Pre	594	29.0	172	24.7	147	29.0	172	11.1	66	6.2	37
		Post	676	26.1	176	22.8	154	25.8	174	15.3	103	10.0	68
14	B	Pre	531	31.0	165	28.2	150	29.7	158	9.7	52	1.4	7
		Post	664	26.6	177	22.2	147	27.4	182	17.9	119	5.9	39
15	B	Pre	309	37.5	116	26.1	81	25.4	78	11.0	34	0	0
		Post	473	30.5	144	18.6	88	24.4	115	11.8	56	14.7	70

FIG. 3.5. CK ISOENZYME COMPOSITION OF SOME HORSE TISSUES



Gels contain (1) Brain
(2) Brain blank
(3) Kidney
(4) Kidney blank
(5) Spleen
(6) Spleen blank

FIG. 3.6. CK ISOENZYME COMPOSITION OF SOME HORSE TISSUES

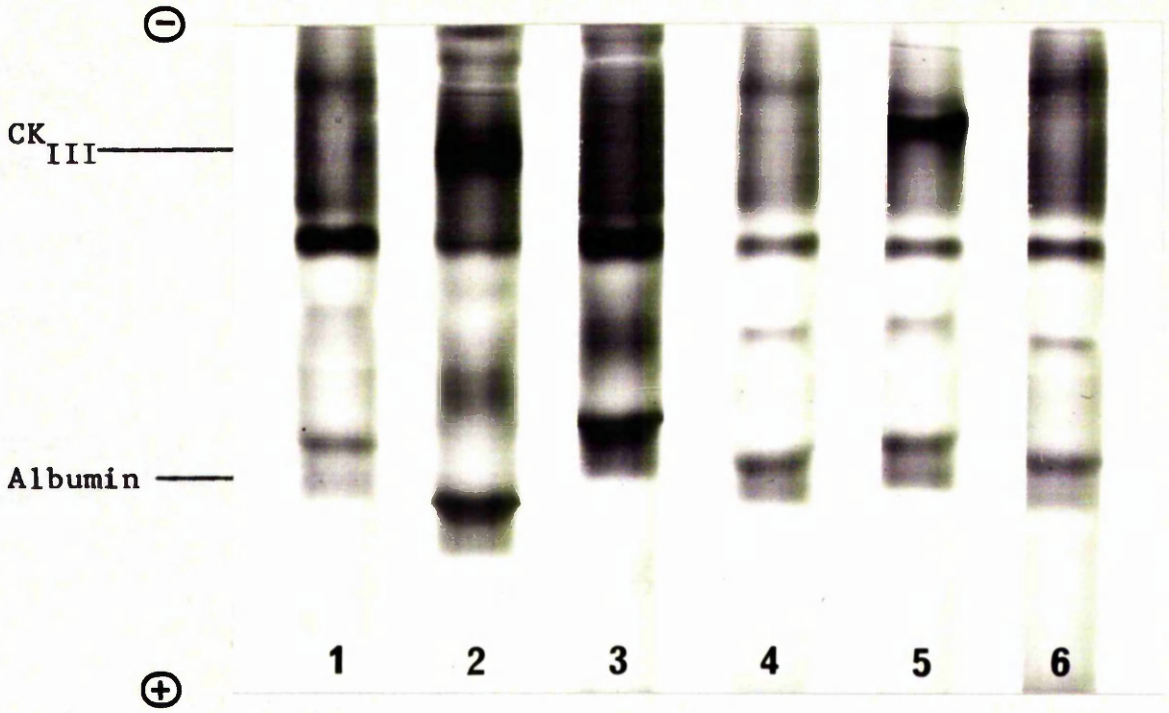


Gels contain (7) Heart
(8) Heart blank
(9) Skeletal muscle
(10) Skeletal muscle blank
(11) Liver
(12) Liver blank

TABLE 3.8. CK ISOENZYME COMPOSITION OF HORSE TISSUES

Tissue	Horse No.	% of Total Activity		
		CK_I	CK_{II}	CK_{III}
Brain	17	100	-	-
	18	100	-	-
Cardiac Muscle	17	-	28	72
	18	-	40	60
Skeletal Muscle (Diaphragm)	17	-	-	100
Skeletal Muscle (Biceps Femoris)	18	-	6	94
Liver	17	100	-	-
	18	100	-	-
Kidney	17	95	-	5
	18	100	-	-
Spleen	17	63	37	-
	18	50	36	14

FIG. 3.7. EFFECT OF EXERCISE ON THE CK ISOENZYME COMPOSITION OF HORSE SERUM



Gels contain serum from (1) Horse 14 before Exercise C
(2) Horse 14 after Exercise C
(3) Horse 14 blank
(4) Horse 15 before Exercise B
(5) Horse 15 after Exercise B
(6) Horse 15 blank.

is CK_I which migrates to the anode side of albumin. Muscle CK activity is composed almost entirely of CK_{III} although 'red' muscles such as biceps femoris also contain some CK_{II}. Cardiac muscle contains both CK_{II} and CK_{III}.

(5) CK isoenzyme patterns in horse serum before and after exercise

No bands of CK activity were observed in any of the sera taken from normal, unexercised horses although the total serum activities varied from 73-116 mI.U/ml. CK isoenzymes were, however, detected in serum taken from animals 5 hours after exercise (Fig. 3.7). In post exercise sera from Horses 14 and 15, a pronounced band of activity was seen in the position of CK_{III}. CK_{II} was not visible in any of the post exercise sera examined.

DISCUSSION

There is relatively good agreement between the present findings (Table 3.1) and those of Gerber (1964, 1965b) regarding the distribution of GOT, LDH, ALD and CK in horse tissues. (No values are given by Gerber for horse erythrocyte enzyme content). The absolute activities, however, in these latter studies are generally lower than the values above. This may be due to the fact that this author carried out enzyme assays by methods using conditions which are not necessarily optimal for the horse enzymes - namely Boehringer Kit Methods for LDH and ALD, the method of Karmen (1955) for GOT and the method of Colombo, Richterich and Rossi (1962) for CK.

One difference exists between the present results and those of Gerber. The concentration of aldolase in horse brain determined in the present study is very much higher relative to the other tissues than in the results of Gerber (1965b) - the reason for this is not known. This high aldolase content probably reflects the dependence of brain on glycolysis as a major metabolic pathway. In contrast, the low aldolase content of horse cardiac muscle suggests that glycolysis is a little used pathway in this tissue, the major sources of energy probably being fatty acids, lactate and possibly glycerol.

The distribution of enzymes in animal tissues is dependent on species. For example, the level of LDH in porcine skeletal muscle is many times higher than in cardiac muscle of the same species (Hyldgaard-Jensen, 1971) while equine heart and skeletal muscle contain similar LDH activities to one another but different from the porcine tissues. In many haemolysis of blood samples results in elevated serum levels of GOT, LDH and ALD (King, 1965). The results above indicate that, of the 4 enzymes under study, only LDH and ALD would be seriously affected by haemolysis in the horse because of the lower erythrocyte/serum GOT activity gradient. CK is seen to be virtually absent from horse erythrocytes in agreement with findings for the human (Solvonuk, McRae and Collier, 1956).

The LDH isoenzyme patterns for equine tissues described above (Table 3.5) are not in complete agreement with the findings of Gerber (1966). One major difference is in the LDH isoenzyme composition of skeletal muscle. The predominant isoenzyme in the present studies is LDH_5 which comprises 36-68% of the total activity while the LDH_5 content of muscle described by Gerber (1966) ranges from 0.14.2% of the total. This may be a reflection of the different functional activities of the muscles examined. The pattern in brain, heart, spleen and kidney is similar in both studies. Comparison of the LDH patterns of erythrocytes obtained

by Coffman et al (1969) with those described above shows identical results.

Gerber observes 6 isoenzymes in horse liver extract, the sixth migrating more slowly than LDH_5. This is in contrast to the findings of Coffman et al (1969) who obtained only 5 bands of activity in agreement with the present studies. It is interesting to speculate on the presence of this extra LDH form.

LDH isoenzymes from vertebrate sources appear to be tetramers composed of two types of subunits named M and H by Kaplan (1964) the synthesis of which is controlled by two independent genes (Markert, 1963, 1964). In addition to the pure isoenzymes M_4 (found mainly in anaerobic tissues such as skeletal muscle) and H_4 (found in aerobic tissues such as heart) there also exist three hybrid forms M_3H, M_2H_2 and MH_3 which are formed by random combination of M and H subunits and which are present to some extent in most tissues. This accounts for the 5 isoenzymes normally seen and the presence of a sixth does not fit the theory easily unless a third separate gene synthesising a third type of subunit C exists. This, however, would be expected to result in more than one additional isoenzyme. Several workers have, in fact, postulated the presence of a third gene as a result of evidence that extra bands of LDH activity exist in extracts of testis and spermatozoa in various species (Zinkham,

Blanco and Kupchyk, 1963; Clausen and Øvlisen, 1965; Valenta, Hyldgaard-Jensen and Moustgaard, 1967). If a third subunit was present in low concentration only one additional hybrid might be seen, most probably M_3C (since liver contains a predominance of M-type subunits), and the electrophoretic mobility of such a hybrid should be between LDH_3 and LDH_5. The extra band of activity found by Gerber (1966) migrated more slowly than LDH_5. Further investigation would be required to determine whether this extra band is an artefact of the method or is, in fact, due to a different subunit structure.

Tissue enzyme patterns vary from species to species (Markert and Møller, 1959). For example, in contrast to the horse and man (Wroblewski and Gregory, 1961) where liver and skeletal muscle patterns are similar, with both tissues containing a large percentage of LDH_5, porcine liver shows a predominance of LDH_3 while porcine skeletal muscle contains mainly LDH_5 (Hyldgaard-Jensen, 1971). Similar species variations are seen in erythrocyte patterns. In the rabbit, guinea pig and dog (Garbus, Highman and Altland, 1967), in man (Wieme, 1959) and in the horse, the predominant form of LDH in erythrocytes is LDH_1 while rat erythrocytes contain mainly LDH_5 (Garbus et al, 1967). In agreement with the findings of Gerber (1966), the results here indicate that the isoenzyme pattern of horse brain contains less H-type

subunits than human brain (Richterich, Gautier, Egli, Zuppinger and Rossi, 1961). Gerber interprets this as meaning that horse brain is less dependent on aerobic metabolism than human brain where glucose is used almost exclusively as a substrate. The high concentration of aldolase found in brain in the present studies suggests that, although horse brain may very well be less critically aerobic than human brain, the main source of energy is still the catabolism of glucose.

The reason for such species variation is unknown since the factors determining the manner in which tissues develop characteristic isoenzyme patterns are not clear. These factors may include (1) different rates of subunit synthesis in response to the biochemical requirements of the cell e.g. M subunits which are not inhibited by the high concentrations of pyruvate found in skeletal muscle during anaerobic glycolysis (Dawson, Goodfriend and Kaplan, 1964) or (2) different rates of release and catabolism of isoenzymes (Fritz, Vessel, White and Pruitt, 1969).

The source of normal serum enzyme activity is difficult to determine for ubiquitous enzymes such as GOT and LDH. Since the serum LDH isoenzyme pattern is a reflection of the various tissue patterns this might be expected to assist the determination of the major contributors to normal serum LDH activity. In the horse, however, the 4 fastest moving isoenzymes are fairly evenly

distributed in serum with LDH_3 the most abundant form (Table 3.6).

When compared with the tissue patterns above, the tissue which has the isoenzyme pattern most resembling the serum pattern is brain. The level of enzyme activity in cerebrospinal fluid under normal conditions is, however, usually low indicating the relative impermeability of brain cells to protein. This tissue is, therefore, unlikely to contribute much to normal serum enzyme activity. The complexity of tissue patterns is such that no single tissue can be picked out as the source of serum LDH activity. Considering the fact that skeletal muscle forms a large part of the body mass, this could be suggested as a major contributor particularly since this tissue contains high concentrations of GOT, LDH, ALD and CK and either skeletal muscle or liver must account for the LDH_4 and LDH_5 found in serum.

The presence of LDH_1 in serum in concentrations ranging from 15-42% indicates that either heart, kidney or erythrocytes should also be contributing. Of these, the possible contribution of erythrocytes can be estimated. The average life span of horse erythrocytes is reported to be 140-150 days (Cornelius, Kaneko, Benson and Wheat, 1960), which means that approximately 0.7% of the total erythrocyte population is destroyed daily. Hof and Woller (1956) observed that venous blood from spleen contains a higher LDH activity than peripheral blood, indicating that LDH

is released from erythrocytes destroyed in the spleen. Assuming the complete release of all the LDH contained in these destroyed cells this would account for 30-40 mI.U/ml. or 5-10% of the total serum activity. In normal serum LDH_1 and LDH_2 combined make up about 50% of the total. Wear and tear on some other tissues rich in LDH_1 and LDH_2, therefore, must account for the remainder. Because of the high erythrocyte/serum ALD gradient, however, erythrocyte destruction may account for about 25% of normal serum ALD activity.

In considering the source of serum LDH, different rates of release of isoenzymes from cells and elimination from the bloodstream must be taken into account. In a comprehensive study on LDH in the pig, Hyldgaard-Jensen (1971) provided evidence that LDH_1 is more readily released from porcine skeletal muscle and liver than LDH_5. Fluid exuding from cut muscles <u>in vitro</u> was found to contain a higher proportion of LDH_1 and LDH_2 than extracts of the same muscles. This worker also showed that LDH_1 has a longer half-life in the blood stream in the pig and on these findings he explained the presence of LDH_1 in serum in higher amounts than would be expected following injury to porcine muscle. Similar observations of different rates of elimination of LDH_1 and LDH_5 have been made in other species including sheep (Boyd, 1967) and man (Fröhlich and Kurrle, 1969). During studies on equine paralytic myoglobinuria, Gerber (1966) found a high proportion of LDH_5 in serum

following an attack but this was replaced within 2 days by a pattern rich in anodic isoenzymes suggesting different elimination rates in the horse also. As a result of these complications, therefore, it is almost impossible to determine the source of normal serum LDH activity on the basis of LDH isoenzyme patterns.

No such difficulty exists for CK isoenzymes since the only band of activity seen in any of the sera examined was CK_{III} the 'skeletal muscle' band. Deul and van Breeman (1964) and Rosalki (1965) both detected only one band of CK activity, the CK_{III} form in human serum from patients with muscular dystrophy. Sjövall and Jergil (1966), on the other hand, demonstrated 4 isoenzymes in serum from a patient with myocardial infarction, 3 in the region of CK_{III} (possibly sub-bands of CK_{III}) and one in the position of CK_{II}.

The difficulty in detection of CK isoenzyme activity in normal serum from resting horses was due to the intense background staining presumably due to sulphydryl groups on proteins. Iodoacetate has no inhibitory effect on horse LDH (personal observation) and can, therefore, be used to reduce the background staining in the separation of LDH isoenzymes. Its use for this purpose on gels to be stained for CK is precluded by its pronounced inhibitory effect on horse CK. This inhibition is in agreement with data for human CK (King, 1965). In these studies glutathione is added to the sample rather than to the incubation medium since

preliminary observations showed that this latter method resulted in the whole gel being stained almost black.

There is a notable lack of information on the distribution of CK isoenzymes in horse tissues and serum. The results described above (Table 3.8) for skeletal muscle, cardiac muscle and brain compare well with findings for human tissues (Deul and van Breeman, 1964; Rosalki, 1965; Trainer and Gruenig, 1968).

In the present studies only CK_{III} was observed in extracts from diaphragm while biceps femoris also contained a trace of CK_{II}. The latter muscle was also seen from the colour of the extract to contain more myoglobin than diaphragm extract. This is of interest since Rosalki (1965), working with human tissues, demonstrated that 'white' muscles contained only CK_{III} while 'red' muscles showed a pattern similar to cardiac muscle containing both CK_{II} and CK_{III}.

Although most work on human tissues has been restricted to heart, skeletal muscle and brain, Trainer and Gruenig (1968) have demonstrated that human thyroid and gall bladder wall contain the same isoenzyme as brain i.e. CK. Van der Veen and Willebrands (1966) similarly illustrated that rat liver, kidney and spleen contain this isoenzyme. The results above show that horse kidney, liver and spleen also contain this band either alone or with small amounts of CK_{II} and CK_{III}.

The significance of the heterogeneity of CK has not been elucidated in the manner of LDH. The H and M subunits of LDH have different substrate affinities allowing them to operate in the presence of different concentrations of pyruvate (Dawson et al, 1964). H type subunits in the heart, therefore, ensure that pyruvate is channelled into the tricarboxylic acid cycle in this tissue while M type subunits convert pyruvate to lactate in tissues such as skeletal muscle which are capable of anaerobic metabolism thereby allowing the regeneration of NAD^+ in the absence of oxygen. No such neat picture has been postulated to account for the physiological significance of CK isoenzymes.

The tissues studied here were chosen because investigations in other species had shown that these tissues are rich in GOT, LDH, ALD and CK and each one is, therefore, a potential source of the serum enzyme increases resulting from exercise. The results above allow this group of tissues to be narrowed.

The very low level of aldolase in horse cardiac muscle (Table 3.1) suggests that it is unlikely that it will contribute greatly to elevations in serum aldolase levels since very considerable cell damage would be necessary before a detectable change in serum aldolase levels would result. The most likely source of aldolase is skeletal muscle which has a very high aldolase content and, therefore, high tissue/serum gradient.

Isoenzyme studies on serum before and after exercise demonstrate that most of the increase in serum LDH is attributable to an increase in LDH_4 and LDH_5 (Table 3.7). From tissue LDH patterns (Table 3.5) these isoenzymes are most likely to originate from skeletal muscle and/or liver (since skeletal muscle contains little LDH_4). It is interesting that Horse 15 shows only an increase in LDH_5 suggesting that in this case only skeletal muscle is involved. The relatively high increase in LDH_1 activity seen in two of the cases might, however, suggest that these are not the only tissues involved.

As discussed earlier, LDH_1 may be more easily released from cells and may have a longer half-life in the bloodstream. In this event, damage to tissues rich in LDH_5 could result in a small but long-lasting rise in serum LDH_1. In the light of these observations, it is quite probable, therefore, that the increase in LDH_1 following exercise is also coming from skeletal muscle. Damage to heart, kidney or erythrocytes would result in a far greater increase in this isoenzyme than is observed here.

In studies on rats, Garbus, Highman and Altland (1964) found that untrained rats subjected to 16 hours exercise showed serum LDH isoenzyme patterns indicating that excess LDH was derived from skeletal muscle, cardiac muscle and other tissues. The severity of the exercise in this study was, however, much

greater than in the present study and this could account for these workers finding increases in anodic LDH isoenzymes indicative of heart involvement. Certainly in the horse there is no strong evidence to suggest that cardiac muscle is a contributor to serum LDH during exercise of the degree used here. On the basis of these studies, a distinction between contributions from liver and skeletal muscle cannot be made.

From the CK isoenzyme studies, however, it is clear that liver plays no part in the increase in serum CK activity during exercise. The total CK content of this tissue is very low and, equally important, the predominant isoenzyme is CK_I (Table 3.8) which has not been seen in any of the sera examined here. Although brain, kidney and spleen contain significant quantities of CK this is also present mainly in the form of CK_I (small percentages of CK_{II} and CK_{III} may be present in kidney and spleen). These tissues, therefore, may be eliminated as likely sources of serum CK. This leaves cardiac muscle or skeletal muscle or both, since more than one tissue may be involved. Distinction is made difficult by the fact that the major component of cardiac muscle CK is CK_{III}, the 'skeletal muscle' form and sera from each horse examined show only this isoenzyme following exercise (Fig. 3.7). On the other hand, although CK_{II} only makes up about 30-40% of the total cardiac muscle CK, this should be visible in the post-exercise

samples if the heart is involved. It is to be expected that changes in membrane permeability which would allow release of CK would also allow release of LDH and ALD. From the discussion above, the heart does not appear to contribute to the serum LDH and ALD increases and, therefore, probably does not contribute to the CK increase either.

In conclusion, therefore, the overall picture suggests that skeletal muscle is the source of the increase in serum ALD, LDH and CK levels resulting from exercise although the liver may contribute to the increase in LDH.

SECTION IV

Effect of Exercise and Training on Blood Metabolites in the Horse

INTRODUCTION

The variations in blood levels of glucose, pyruvate and lactate during muscular activity in man have been extensively studied (Christensen and Hansen, 1939; Huckabee, 1958b; Johnson, Walton, Krebs and Williamson, 1969). High concentrations of blood lactate and pyruvate have been observed following exercise, particularly in untrained individuals. Changes in blood glucose levels are less consistent. The effects of exercise on lipid metabolism in man are also well documented (Carlson and Pernow, 1961; Havel, Naimark and Borchgrevink, 1963; Johnson et al, 1969). An initial decrease in plasma free fatty acids (FFA), due to increased efflux from the plasma, is followed by an increase in FFA as a result of increased mobilisation from adipose tissue. High blood glycerol concentrations, indicative of increased fat mobilisation, are also observed during exercise.

In contrast, comparatively little is known of carbohydrate and lipid metabolism during exercise in other species although some studies have been made in the rat (Gollnick, 1967) and in the dog (Issekutz, Miller, Paul and Rodahl, 1965; Issekutz and Paul, 1966).

Surprisingly, relatively few studies have been made on

the changes in blood metabolites i.e glucose, lactate, pyruvate, FFA, glycerol and ketone bodies in the horse - a species adapted for running. Of particular interest is the relative importance of lipid and carbohydrate metabolism during exercise in this species. Carlson, Froberg and Persson (1965), studied the concentration of glucose and the concentration and turnover of FFA in the plasma of horses during exercise but this study was restricted to changes during trotting and cantering and did not include the effects of galloping. Asheim, Knudsen, Lindholm, Rülcker and Saltin (1970) have measured blood lactate levels in the horse during exercise.

In the present study, the effects of exercise of different intensities on blood concentrations of glucose, lactate, pyruvate, lactate/pyruvate ratio, FFA and glycerol were studied together with the effects of training.

Prior to any of these studies, however, the effect of diet on each of these parameters had to be estimated to determine whether feeding the animal before exercise would interfere with or mask the metabolic effects of exercise.

In the past, the relationship between blood lactate and pyruvate levels has been used in man as an index of the oxygen deficiency of the tissues. This is based on the findings of Huckabee (1958a and b) who pointed out that blood lactate

concentrations vary with both pyruvate concentration and oxygen deficiency in accordance with the equation:

$$[\text{lactate}] = [\text{pyruvate}] \times K \frac{[NADH]}{[NAD^+]}$$ where K is a dissociation constant.

The validity of using the lactate/pyruvate ratio as an index of the degree of tissue hypoxia will be discussed later. In the present study it should be possible on this basis to distinguish between aerobic and anaerobic metabolism during exercise in the horse.

MATERIALS AND METHODS

Materials

The chemicals used in this section are listed below. Unless otherwise stated, all reagents are "Analar" grade from BDH Chemicals Ltd.

For glucose estimation

Ammonium molybdate.

Benzoic acid.

Cupric sulphate, hydrated.

D(+) Glucose.

Sodium arsenate.

Sodium bicarbonate.

Sodium carbonate, anhydrous.

Sodium potassium tartrate.

Sodium sulphate.

Sodium tungstate.

Sulphuric acid, concentrated.

For lactate estimation

Lactic acid, standard solution, (Sigma Chemical Company Ltd.)

Biochemica Test Combination for Lactate Estimations - Cat. No. 15972 from the Boehringer Corpn. (London) Ltd., London, containing:

0.5M-glycine buffer, pH 9.0; 0.4M-hydrazine

LDH (2 mg/ml)

0.027M-NAD.

For pyruvate and acetoacetate estimation

Ethyl acetoacetate.

Hydroxybutyrate dehydrogenase (Boehringer Corpn. Ltd.)

Lactate dehydrogenase (Boehringer Corpn. Ltd.)

Malate dehydrogenase (Boehringer Corpn. Ltd.)

β-Nicotinamide adenine dinucleotide, reduced form (NADH).
(Boehringer Corpn. Ltd.)

Perchloric acid.

Pyruvic acid, standard solution (Sigma Chemical Co. Ltd.)

Tripotassium orthophosphate.

For glycerol estimation

Glycerol.

Biochemica Test Combination for estimation of neutral fat and glycerol - Cat. No. 15989 from the Boehringer Corpn. Ltd. containing:

0.1M-triethanolamine buffer, pH 7.6; 4mM-magnesium sulphate. 6mM-NADH; 33mM-ATP; 11mM-PEP

LDH (2 mg/ml); Pyruvate kinase (1 mg/ml)

Glycerokinase (2 mg/ml)

For free fatty acid estimation

n-Butanol (May & Baker Ltd., Dagenham)

Chloroform.

Cupric nitrate, hydrated.

Palmitic acid, specially pure.

Potassium dihydrogen orthophosphate.

Sodium diethyldithiocarbamate.

Sodium hydroxide.

Triethanolamine hydrochloride (Sigma Chemical Co. Ltd.)

Methods

Collection and preparation of blood from the horse for metabolite estimations

It is essential that blood be processed immediately on sampling since blood levels of metabolites alter very rapidly (within minutes).

20 ml. of blood withdrawn from the jugular vein was divided as follows:-

For glucose estimation - approximately 1 ml. was added to a tube containing sodium fluoride and sodium citrate and mixed immediately.

For lactate, pyruvate and acetoacetate estimations - 10 ml. of blood were deproteinised by pipetting into a 50 ml. centrifuge tube containing 10 ml. of 0.7M cold perchloric acid, mixing the contents and centrifuging at 2,200 g. for 10 minutes. The supernatant was then used for lactate, pyruvate and acetoacetate estimations.

For free fatty acid and glycerol estimations - the remainder of the blood was collected in a plain polystyrene tube and serum prepared. This material may also be used for serum enzyme measurements.

It is important that blood glucose, free fatty acid and glycerol estimations be carried out within a few hours of sampling.

For blood pCO_2 and bicarbonate measurements - 2-3 ml. of blood were collected in a heparinised syringe and the needle tip sealed with a rubber stopper to prevent loss of carbon dioxide to the atmosphere. The syringe was then kept in ice until the determinations were made (not later than 3-4 hours after sampling).

Estimation of blood glucose, lactate, pyruvate, acetoacetate, free fatty acid and glycerol levels

All measurements were made in duplicate.

Blood glucose levels were measured colorimetrically by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1945). Extinction readings were made on a Unicam SP600 spectrophotometer.

Blood lactate levels were estimated using the Biochemica Test Combination Cat. No. 15972 (Boehringer, 1969) which is based on the enzymatic method of Hohorst, Kreutz and Bücher (1959). This method was found to be linear up to a lactic concentration of at least 160 mg/100 ml. blood and the presence of a high concentration of pyruvate (16 mg%) did not interfere with the estimation. Extinction readings at 340 nm. were made on a Unicam SP800 spectrophotometer. If the extinction change exceeded 1.5 Units the sample was diluted with 0.5M-glycine buffer, pH 9.0, and the assay repeated. With each batch of samples a blank and a lactate standard containing 0.40 mg/ml. were included.

Blood pyruvate and acetoacetate levels were assayed using

a method for the simultaneous enzymatic determination of aceto-acetate and pyruvate (Bergmeyer, 1965). The method was found to be linear up to a pyruvate concentration of at least 4 mg% and an acetoacetate concentration of up to 2 g%. Since, however, several of the post-exercise samples examined in the present study showed pyruvate levels of 3-4 mg%, the concentration of the NADH solution used in the method was increased from 6 mg/ml. to 7 mg/ml. to allow a greater extinction change to take place and, therefore, to widen the range of the assay. If the extinction change exceeded 1 unit, however, the sample was diluted with 0.1M-phosphate buffer, pH 7.0-7.4 and the assay repeated. With each batch of samples a blank and a standard were included.

Serum glycerol levels were estimated using the Biochemica Test Combination Cat. No. 15989 (Boehringer, 1969) which is based on the enzymatic method of Eggstein and Kreutz (1966). This method was found to be linear up to a glycerol concentration of at least 10 mg/100 ml. serum. With each batch of samples a blank and a glycerol standard containing 1 mg/100 ml. were run.

Free fatty acids (FFA) in serum were estimated using the colorimetric procedure of Itaya and Ui (1965). This method involves the extraction of the free fatty acids with chloroform and phosphate buffer (to reduce the extraction of phospholipids) followed by the formation of copper-fatty acid salts and the

estimation of the resulting copper present in the chloroform as described by Duncombe (1963). One minor modification was made - the last traces of cupric aqueous layer were removed by passing through Whatman phase separating paper, thus avoiding contamination of the chloroform layer. The method was shown to be linear up to a FFA concentration of at least 3.0 mEq/l.

When applying this method to horse serum, some interference may arise owing to the presence in horse serum of high concentrations of bilirubin and similar pigments which are extracted into the chloroform layer and which absorb at the same wavelengths as the copper-fatty acid salts. This can be compensated for by making up a chloroform blank for such sera (prepared by extracting the serum with chloroform as usual and drying by passing through phase separating paper).

Blood pH and pCO_2 measurements were made using the Blood Micro System Type BMS3 and Digital Acid-Base Analyzer PHM72 (Radiometer Ltd., Copenhagen). This system incorporates a pH electrode, a pO_2 electrode and a pCO_2 electrode. Blood bicarbonate and base excess were determined from the Siggaard-Andersen Alignment Nomogram (1963).

Exercise programmes

In this section the exercises described in Section II were performed. For comparative purposes, the paces trot, canter and gallop were characterised by the following speeds.

During trotting, the speed ranged from 3-5 metres/second depending on the horse. Similarly, during cantering and galloping, the average speeds were 5.2-6.7 m/sec. and 11.0-13.3 m/sec. respectively, again depending on the horse. In each case, however, the gallop speed was associated with the maximum effort of the horse.

RESULTS

(1) Effect of feeding on blood levels of glucose, lactate, pyruvate, free fatty acids and glycerol in the horse

The effect of a meal containing 2 kg. pony cubes, 0.75 kg. bran and 8 kg. hay and one consisting of 8 kg. hay alone were compared. At the beginning of each experiment the animals were in a fasting condition, the last meal having been taken the previous evening (17-18 hours before) although in a few cases some hay remained from this meal.

Blood samples were taken before feeding and then at 30-45 minute intervals for 3-5 hours. Glucose, lactate and pyruvate estimations were carried out on blood from 3 horses and typical results are shown in Fig. 4.1. This shows that blood glucose levels remain fairly constant when the animal has access to hay alone but, when the complete meal is provided, a glucose peak is observed. Blood pyruvate and lactate levels show a slight increase throughout the morning irrespective of which meal is provided. Free fatty acid and glycerol estimations were also carried out on serum from 3 horses. Both these metabolites appear to decrease in concentration during the morning irrespective of the food ingested (Fig. 4.2 shows results for one horse) although one animal showed constant glycerol levels after a complete meal.

FIG.4.1 EFFECT OF DIET ON THE CONCENTRATION OF GLUCOSE, LACTATE AND PYRUVATE IN BLOOD

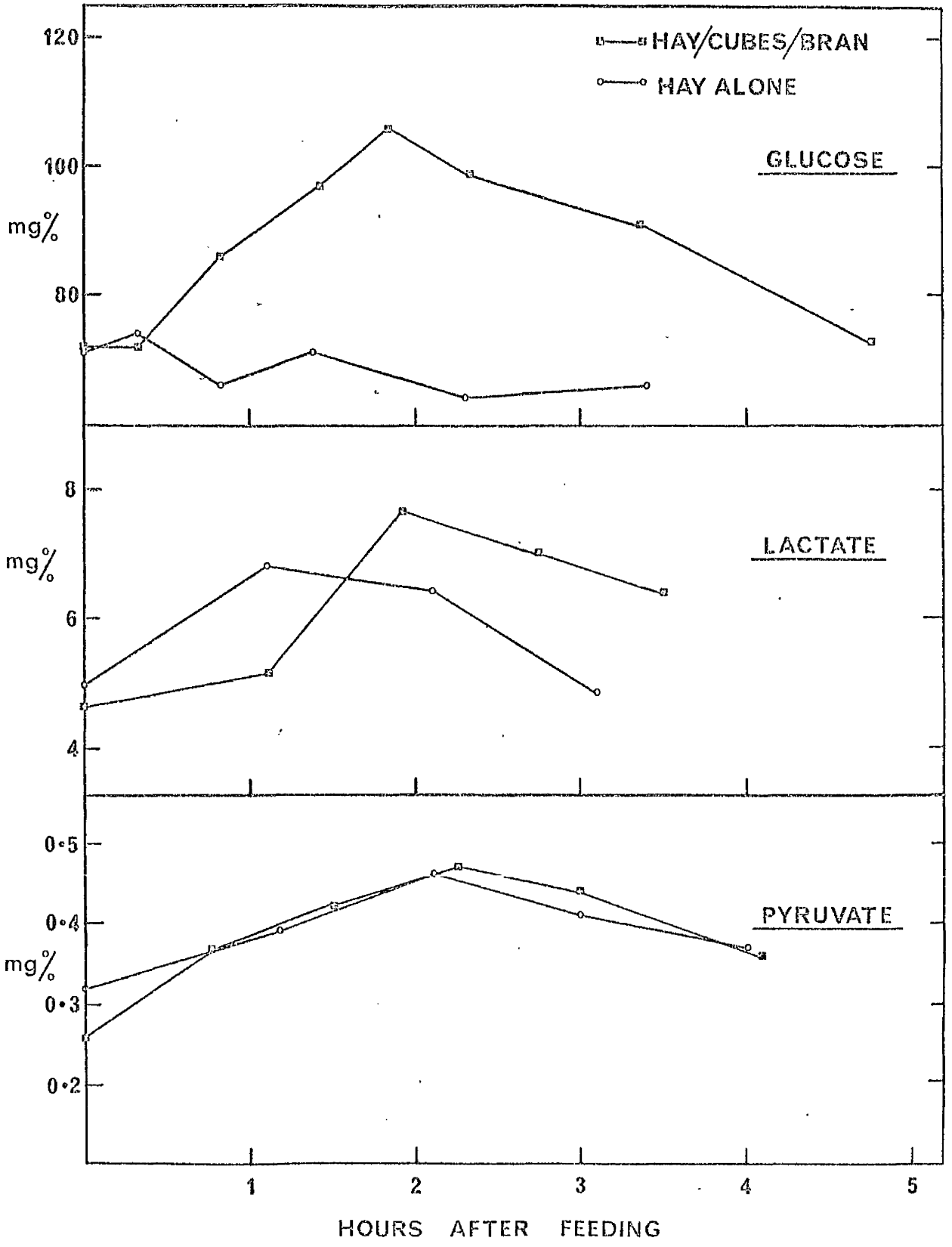
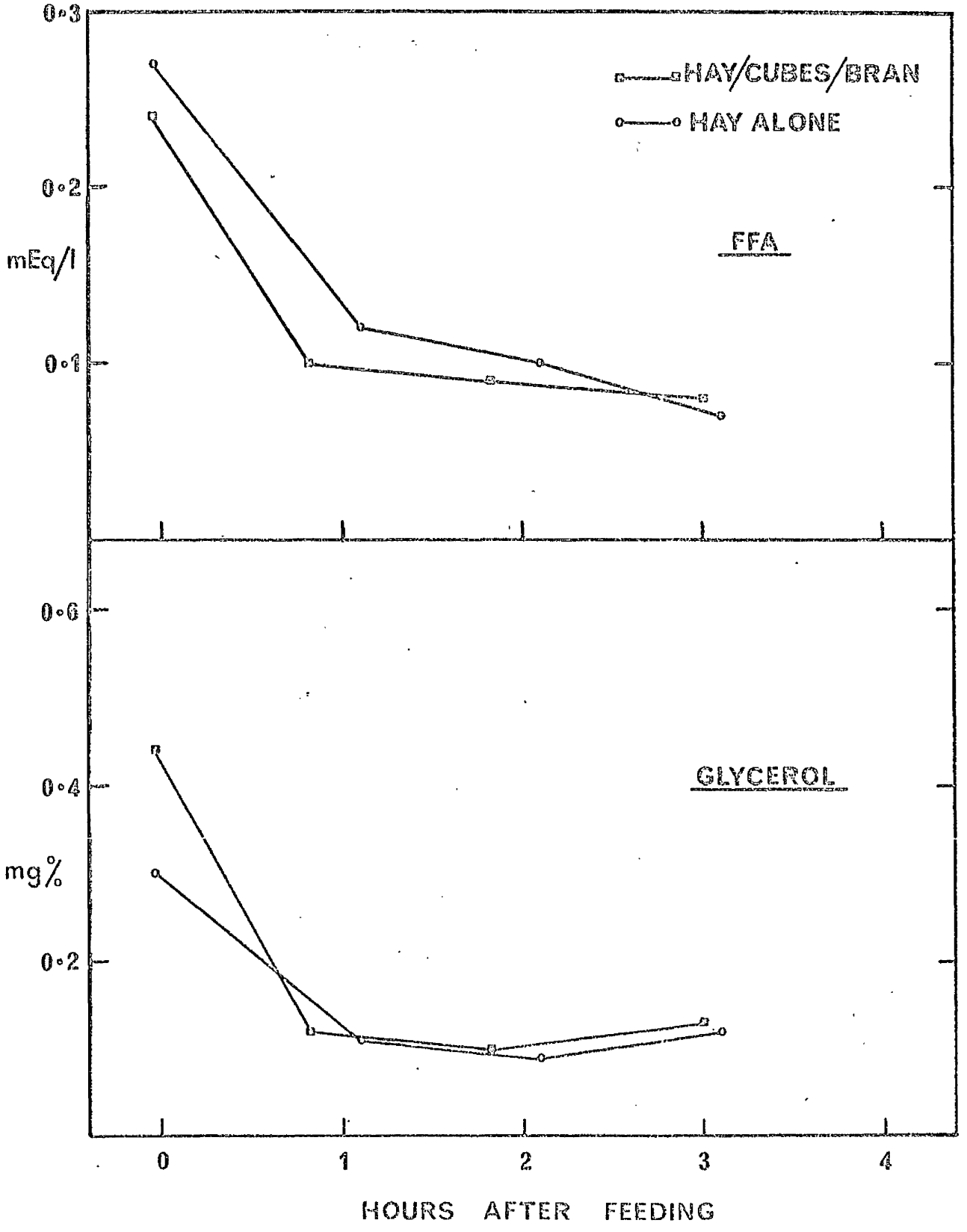


FIG. 4.2. EFFECT OF DIET ON THE CONCENTRATION OF FREE FATTY ACIDS AND GLYCEROL IN SERUM



On the basis of these findings, all subsequent experiments were performed on horses which had the main portion of their morning meal withheld and had access only to hay. In the present studies, such animals are referred to as "fasting".

(2) Normal levels of blood metabolites in the "fasting" horse

Normal levels of blood glucose, lactate, pyruvate, free fatty acids and glycerol were measured in 9 different horses on several different occasions. In each case, samples were taken in the early morning before the animals were exercised or fed (as close as possible to basal conditions). Table 4.1 lists the findings together with data obtained from the literature for man. In general, the normal values are similar for both species. Acetoacetate was not detected in most of the horse sera examined.

(3) Effect of exercise on blood metabolites in the horse

Blood glucose, lactate and pyruvate levels were monitored before, during and immediately after exercise in 7 horses subjected to either Exercise A or Exercise B (each horse being tested 3 or 4 times). In all cases, blood lactate and pyruvate levels rose dramatically during exercise, particularly during galloping, and began to fall immediately the exercise ceased. Fig. 4.3 shows the effect on 2 horses exposed to Exercise B. This figure includes the lactate/pyruvate ratio and shows that this also

TABLE 4.1. NORMAL RANGE OF BLOOD METABOLITES IN THE RESTING, FASTING HORSE

Metabolite	No. of Samples	Mean	S.D.	Range	Normal Values for Human
Glucose (mg%)	44	73	± 8.35	(54 - 86)	65 - 110 [a]
Lactate (mg%)	43	5.5	± 1.76	(2.8-11.0)	9 - 16 [b]
Pyruvate (mg%)	41	0.50	± 0.19	(0.24 - 1.28)	0.36 - 0.59 [c]
Lactate/Pyruvate Ratio	39	11.4	± 3.01	(4.7 - 17.8)	
F.F.A. (mEq/l)	32	0.23	± 0.14	(0.03 - 0.49)	0.09 - 0.6 [d]
Glycerol (mg%)	30	0.55	± 0.25	(0.25 - 1.43)	0.5 - 1.7 [e]

a Hawk's Physiological Chemistry (1965)

b Landahn (1959)

c Landon, Fawcett and Wynn (1962)

d Howorth, Gibbard and Marks (1966)

e Eggstein (1966)

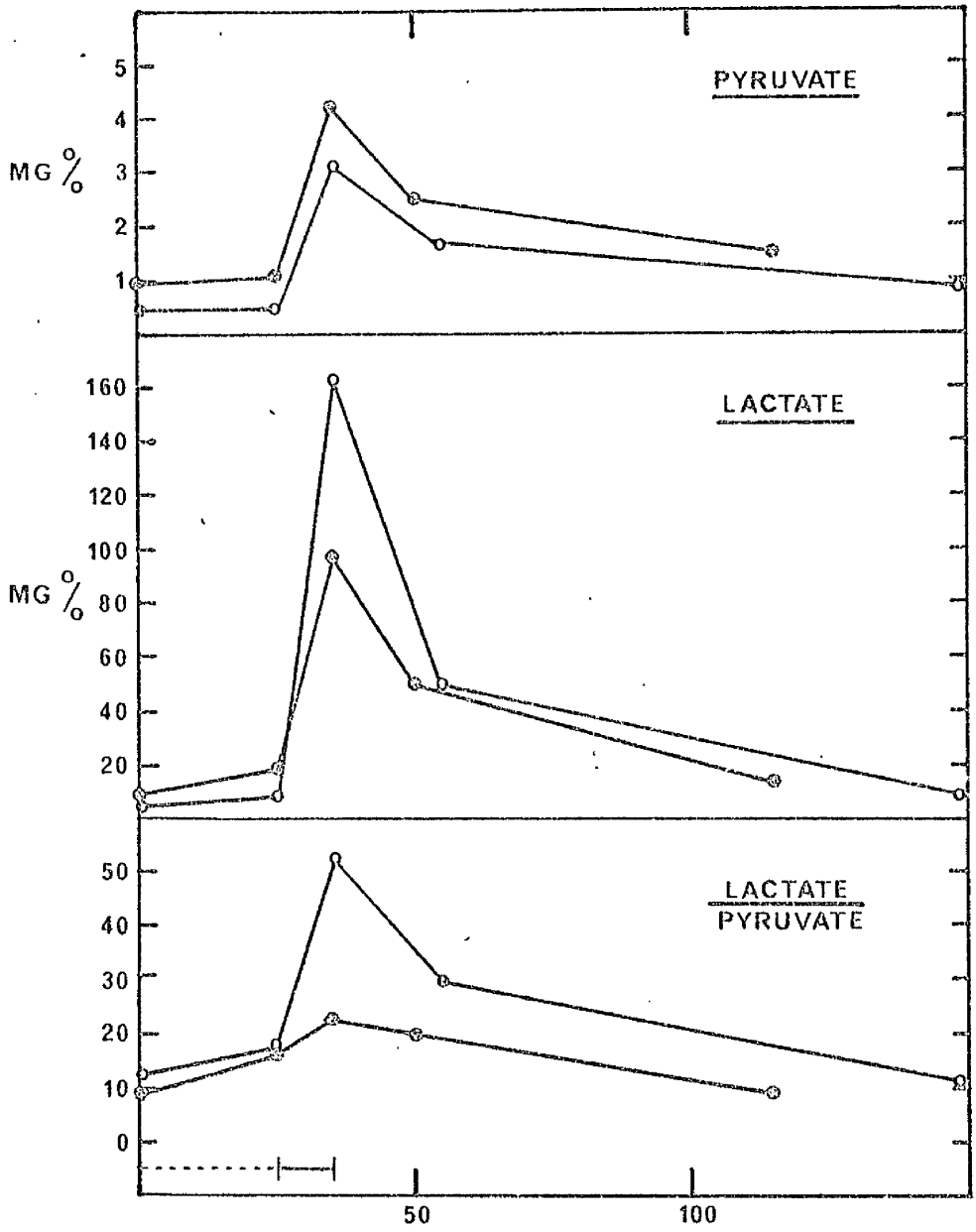


FIG. 4.3 EFFECT OF EXERCISE ON BLOOD LACTATE, PYRUVATE AND LACTATE:PYRUVATE RATIO.

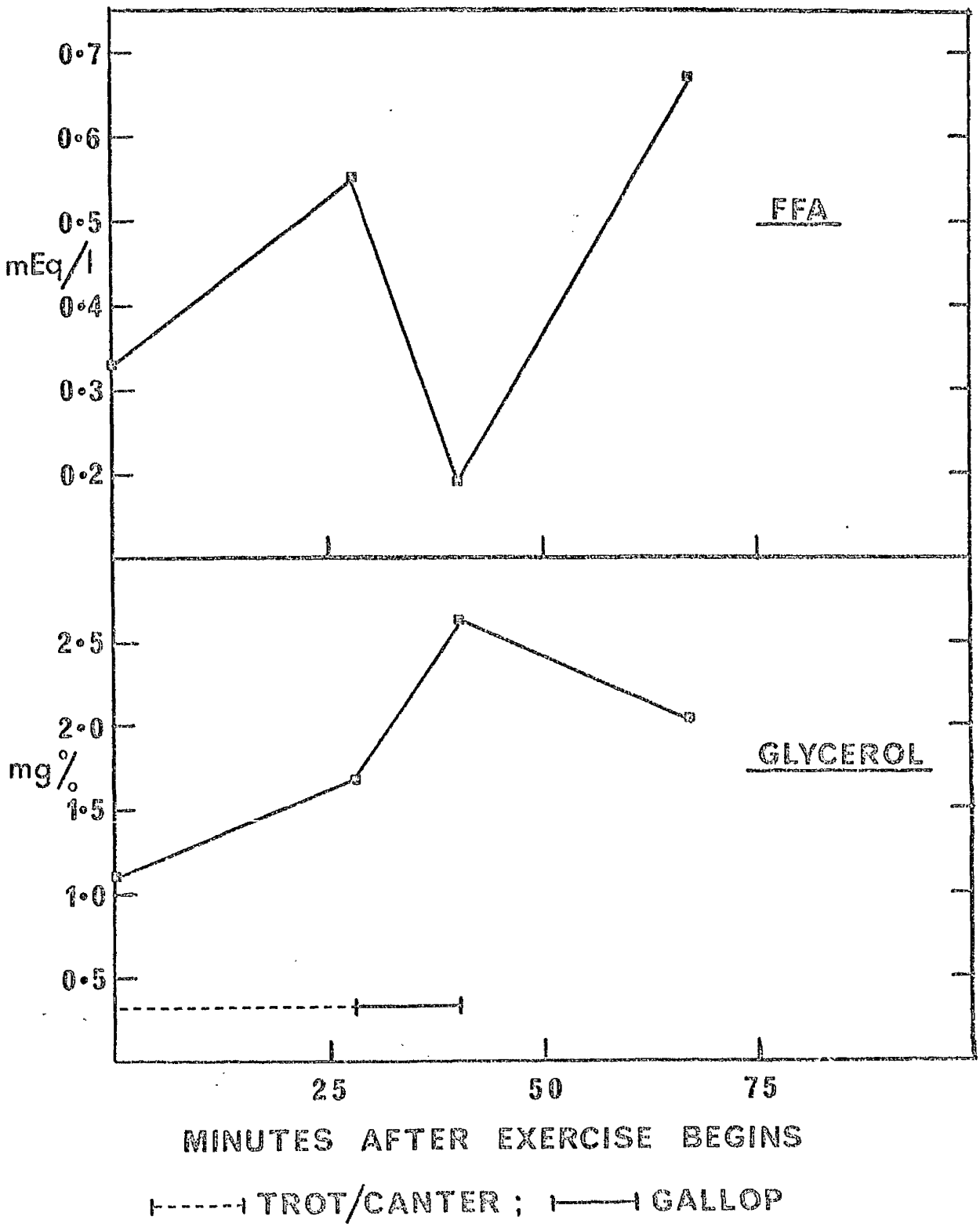
increased during exercise.

The changes in blood glucose levels were less consistent with some horses showing an increase while others remained constant. In most of those animals which showed an increase, a slight initial decrease in blood glucose was observed, followed by the increase. Fig. 4.4 shows the effect of Exercise B on serum free fatty acids and glycerol for one horse. Glycerol levels increased during exercise and remained elevated for some time after the exercise had ceased. FFA levels, on the other hand, increased initially and then decreased as the intensity of the exercise also increased. When the exercise ceased, FFA levels rose again.

(4) Effect of training of blood metabolites in the horse

When the horses were exposed repeatedly (at weekly intervals) to Exercise B and blood metabolite levels measured before, during and after exercise on each occasion, no significant effect was observed on the changes in these metabolites resulting from such exercise, i.e. no training effect was seen. An alternative form of exercise namely daily trotting and cantering for 13 km over a 3 week interval was undertaken by one horse. Blood samples for metabolite assay were taken before and immediately after exercise on the 1st, 8th and 16th day of the programme. In this case, the effects shown in Fig. 4.5 were observed, i.e. the increases in lactate, pyruvate and lactate/pyruvate ratio decreased

FIG. 4.4. EFFECT OF EXERCISE ON THE CONCENTRATION OF FREE FATTY ACIDS AND GLYCEROL IN SERUM



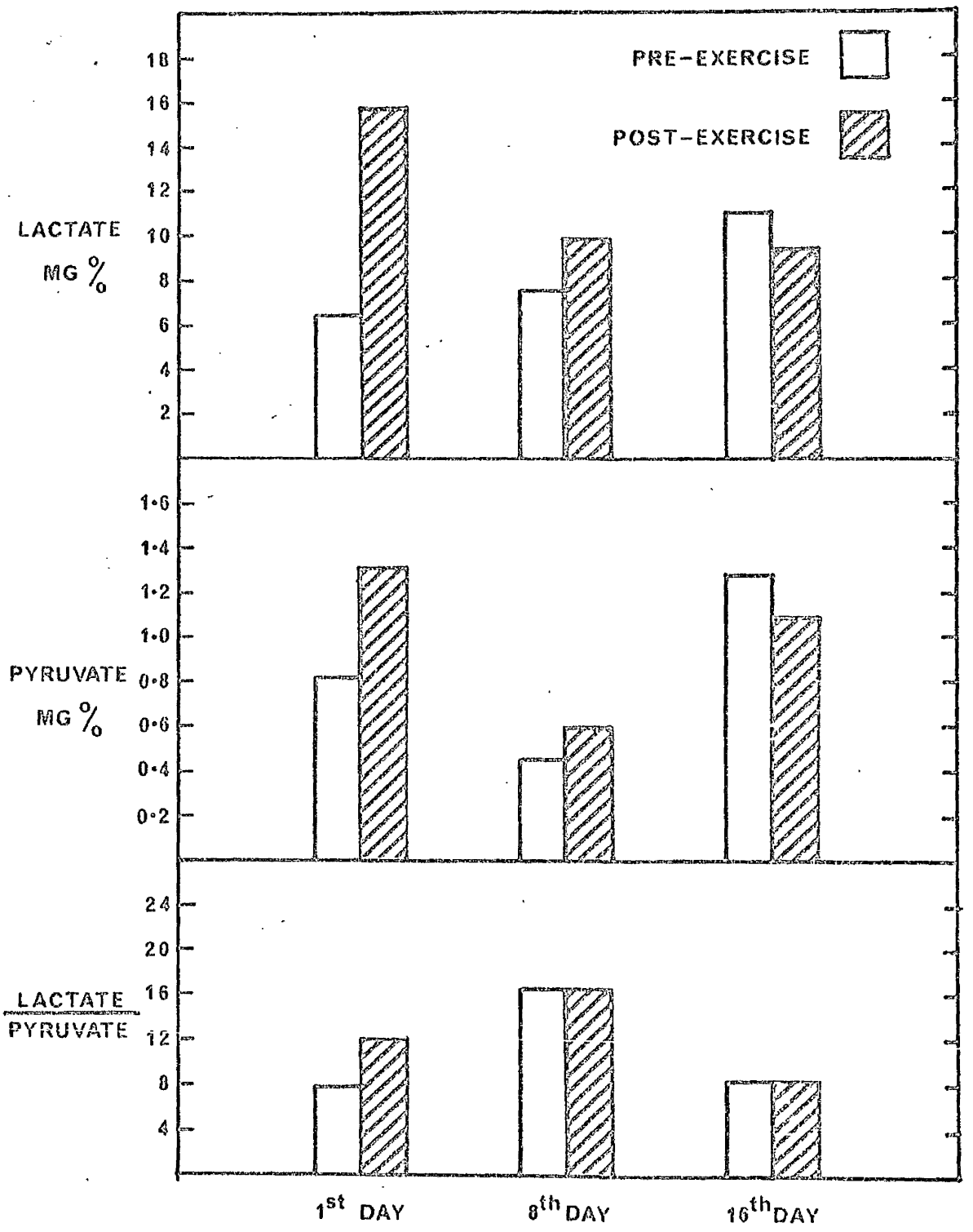


FIG. 4.5 EFFECT OF TRAINING ON BLOOD LACTATE, PYRUVATE AND LACTATE : PYRUVATE RATIO.

over the 3 week period. The animal was, therefore, showing a training effect when subjected to exercise daily rather than once weekly.

(5) Effect of exercise intensity on blood metabolite levels

The effect of varying the intensity of exercise on metabolites was also examined. When 4 horses were subjected to Exercise B (trot/canter/gallop) and also to Exercise C (trot/canter), it became obvious that the pace or intensity of the exercise was the major factor determining blood metabolite levels. A fifth horse was also made to canter for 4.2 km (6.7 metres/sec) and, on a different day, the same distance at the gallop (11.8 metres/sec). Table 4.2 compares the effect on the various metabolite levels of these two paces. Blood lactate, pyruvate and lactate/pyruvate ratio increase dramatically during the gallop in each case. During cantering, on the other hand, there is little change in these parameters. Changes in blood glucose levels are less dramatic although there appears to be a greater tendency for blood glucose to increase during the gallop. A severe metabolic acidosis, indicated by a large negative Base Excess and pH change in Table 4.2, develops during galloping. This is probably largely due to excessive lactate production.

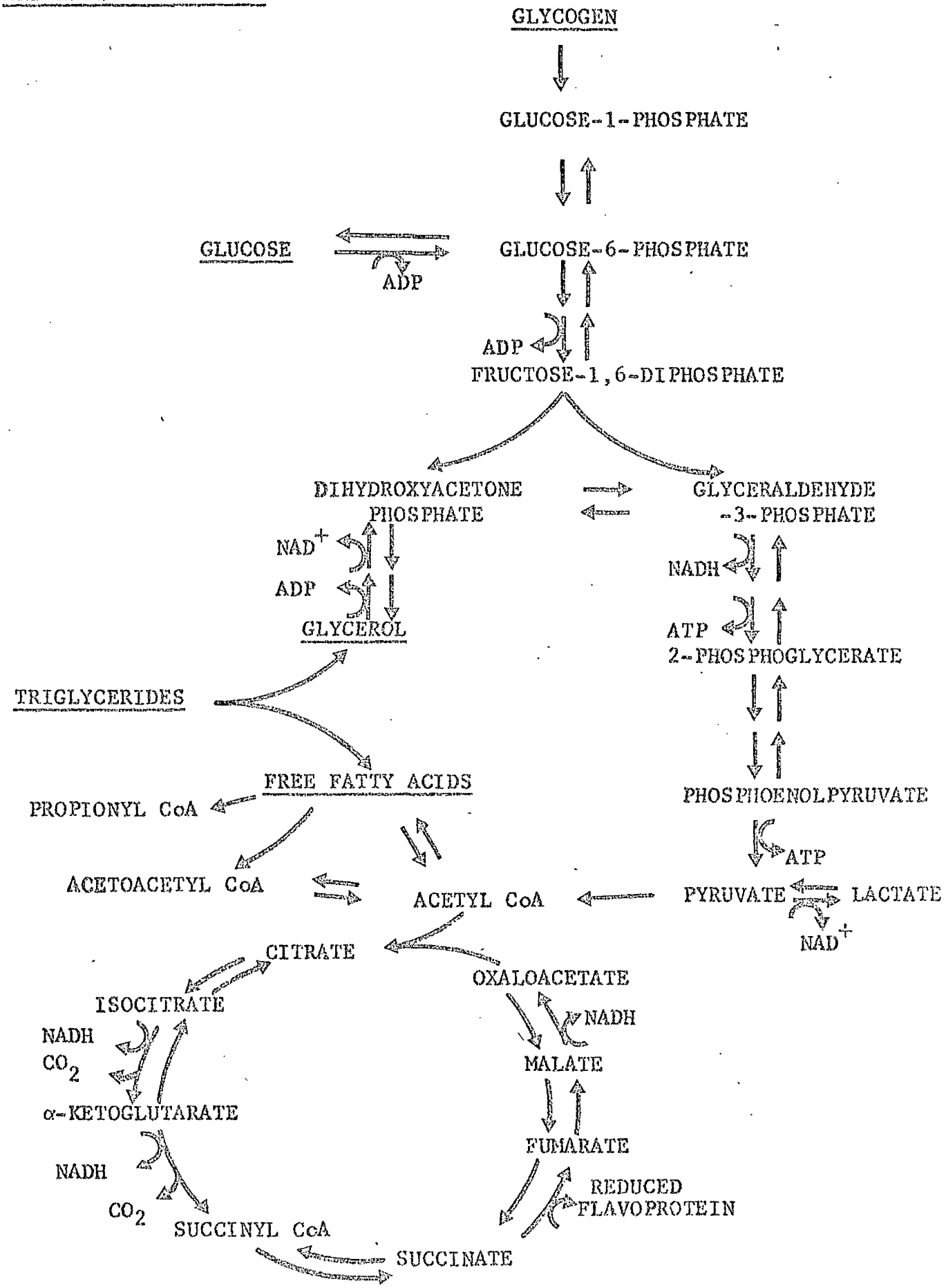
Body lipids are mobilised to a similar extent at both paces (indicated by similar increases in blood glycerol levels).

TABLE 4.2. EFFECT OF EXERCISE INTENSITY ON BLOOD METABOLITE LEVELS IN THE HORSE

Horse No.	Pace	Increase in			Change in				
		Lactate mg%	Pyruvate mg%	Lactate/ Pyruvate mg%	Blood pH	Base Excess mEq/1	Glucose mg%	FFA mEq/1	Glycerol mg%
3	Canter	9.8	0.03	19.7	-	-	+ 15	-	-
	Gallop	136.5	4.00	17.8	-	-	+ 33	-	-
6	Canter	3.5	0.27	2.6	+ 0.005	- 3.9	- 9	+ 0.49	+ 1.99
	Gallop	107.7	4.53	11.5	- 0.135	- 9.1	- 4	- 0.14	+ 1.52
12	Canter	5.1	0.06	8.2	-	-	- 24	+ 0.25	+ 0.96
	Gallop	61.9	1.20	25.8	-	-	+ 7	-	-
14	Canter	4.8	0.48	0.9	+ 0.009	0	- 9	+ 0.03	+ 1.01
	Gallop	127.7	2.36	35.8	- 0.106	- 14.9	+ 7	+ 0.05	+ 1.23
15	Canter	4.5	0.31	1.8	-	-	+ 2	+ 0.09	+ 0.21
	Gallop	146.4	2.07	49.0	-	-	- 7	- 0.03	+ 0.90

The behaviour of serum FFA levels may, however, differ greatly at each pace. The results in Table 4.2 do not present the complete picture and the effect of pace on FFA levels is better illustrated in Fig. 4.4. Here it is seen that FFA levels increase during trotting and cantering but once the pace is increased to a gallop, the FFA levels fall.

FIG. 4.6. MAIN METABOLIC PATHWAYS FOR THE OXIDATION OF CARBOHYDRATES AND LIPIDS IN MUSCLE



DISCUSSION

There are several possible energy sources readily available for use by muscles. These include glucose stored as glycogen within the muscle and available from the liver, free fatty acids and glycerol from adipose tissue and amino acids from the amino acid pool. In the absence of severe malnutrition, however, amino acids can be discounted as a likely fuel during short term exercise. As long ago as 1866, Pettenkofer and Voit showed that protein combustion during heavy exercise was no higher than under resting conditions and this finding has frequently been confirmed since.

Lactic acid can also act as a fuel for some tissues e.g. heart, kidney during exercise. These tissues normally have an adequate supply of oxygen at all times. During mild, aerobic exercise skeletal muscle could use lactate as a fuel.

It is now known that, in the human at least, the metabolism of muscle can be influenced to some extent by the diet. In 1911, Zuntz found very low values for the respiratory quotient (RQ) suggesting an almost exclusive fat metabolism in men participating in mild exercise after an extremely fat-rich diet. These findings have since been confirmed and extended by Christensen

and Hansen (1939) who showed that subjects living on a high-fat diet were exhausted more quickly and had a lower RQ than subjects living on a normal or very high-carbohydrate diet. These workers concluded, therefore, that the diet affects the relative participation of fat and carbohydrate in energy metabolism.

Another important conclusion derived from this latter work was that the contribution of fat or carbohydrate to energy metabolism depends on the intensity of the work. When subjects fed on a normal diet participated in moderate exercise, 50-66% of the energy was supplied by fat. When these subjects took part in exercise of such an intensity that anaerobic metabolism was involved, most of the energy was provided by carbohydrate. It appears that the closer the subject works to his maximum effort, the more important is carbohydrate as a fuel.

These investigations of Christensen and Hansen were carried out on the human and were based on the measurement of RQ values. The one drawback of this method is that, during heavy exercise, lactate is produced and the resulting metabolic acidosis causes the subject to hyperventilate. In consequence, the RQ rises because carbon dioxide is being blown off. In addition, although practical in man, the measurement of RQ values is not easily applied to the exercising horse.

From the results obtained in the present studies,

however, some qualitative conclusions can be drawn regarding the metabolism of fats and carbohydrate in the horse during exercise. First the diet must be considered. The animals used in the present study were maintained on a well-balanced diet - therefore the diet would not be expected to influence the fuel used during exercise. On the other hand, the digestive tract in the horse differs from that in man in the use of the caecum and large colon for fermentation. In these organs, dietary polysaccharides such as cellulose, contained mostly in hay and grass, are fermented microbially to volatile fatty acids (mainly acetic, propionic and butyric acids) in a manner similar to that found in the rumen. These short chain fatty acids are readily absorbed from the caecum and colon (Barcroft, McAnally and Phillipson, 1944) and may act as precursors for the synthesis of the long chain fatty acids (chain length C_{14}-C_{22}) which, as triglycerides, constitute the neutral fat of adipose tissue, or, in the case of propionate, this may be used for the synthesis of glucose. Whether this results in an increase in the importance of free fatty acids as an energy source for muscle in the horse, in analogy with the dietary influence observed in man, is not known.

The circulating levels of free fatty acids (FFA) and glycerol determined above for the resting, fasting horse are similar to those found in the human under resting conditions.

(Table 4.1). It should be noted, however, that colorimetric methods for the estimation of FFA such as that used here, only measure long chain fatty acids (chain length from C_{10} upwards). Volatile fatty acids in the bloodstream will not be detected. In the present studies, however, the FFA of interest are those which are mobilised from the body fat depots in response to stimuli and these are all long chain. Fasting levels of blood glucose in the horse appear to be lower than those found in the human but higher than those found in the ruminant - an observation previously made by Alexander (1955). Blood lactate and pyruvate levels are similar to those found in the human. The fact that blood lactate levels and more particularly blood FFA levels compare well with those obtained for the resting, fasting human indicates that sampling in these horses does not produce undue excitement or agitation and that the animals are in a reasonably basal state at sampling.

The influence of diet on blood metabolites is reported here. A peak in blood glucose levels is seen about 2 hours after the provision of concentrates plus hay but not after hay alone (Fig. 4.1). In contrast, blood glucose levels in the human reach a peak within 1 hour of oral administration of glucose. This difference may reflect the time required for ingested glucose to reach its site of absorption - the ileum in both species. The

absence of a glucose peak following a meal of hay alone is probably due to the continual fermentation of the polysaccharides in this material to volatile fatty acids in the caecum. Blood lactate and pyruvate levels increase slightly over the morning irrespective of the food provided. Alexander and Davies (1963) reported that lactic acid is produced in the horse stomach as a result of fermentation and that large quantities of lactate are absorbed from the small intestine. This would account for the increase in blood lactate and pyruvate levels (since the two are in equilibrium) following ingestion of hay. Another possibility is the increased utilisation of glucose during the morning. This would also result in small elevations in pyruvate and consequently lactate levels. Increases of 20-50% in blood lactate and pyruvate are observed following breakfast in the human (Friedemann, Haugen and Kmieciak, 1945).

The mobilisation of lipids (Fig. 4.2) appears to be somewhat depressed during the morning (indicated by the decrease in both FFA and glycerol levels). The reasons for this are unclear. That it is not related to the changes in blood glucose is obvious from the fact that the effect is seen with both diets. Since increased blood lactate levels are known to depress lipid mobilisation in dogs (Issekutz and Miller, 1962), the effect may be attributable to a similar response in the horse.

As described above, the effect of exercise on blood glucose levels in the horse is variable with some animals showing an increase, some a decrease etc. (Table 4.2). The blood glucose level itself does not provide much information on carbohydrate metabolism during exercise since this level reflects both the uptake of glucose by working tissues and its replenishment by hepatic glycogenolysis and depends, therefore, on the balance between these two processes. When blood glucose levels increase or remain constant during exercise this does not necessarily indicate a decreased utilisation of glucose by muscle but more probably reflects the efficiency of the homeostatic mechanisms. With this in mind, it is interesting to note that during studies in man, Johnson et al (1969) observed increases in blood glucose levels during exercise in trained athletes while untrained individuals showed a slight decrease.

The product of glucose metabolism in working muscle is pyruvate which, in the presence of oxygen, is completely oxidised to carbon dioxide and water via the tricarboxylic acid cycle. In the absence of sufficient oxygen, however, lactate is formed from pyruvate (Fig. 4.6). In extensive investigations into the production of lactate and pyruvate in man, Huckabee (1958a and b) showed, using pyruvate perfusions, that, in the absence of an oxygen deficiency, blood lactate levels rise in

proportion to pyruvate levels. This is in agreement with the equation -

$$[\text{lactate}] = [\text{pyruvate}] \times K \frac{[\text{NADH}]}{[\text{NAD}^+]}$$ where K is a dissociation constant.

When there is insufficient oxygen present the ratio $\frac{\text{NADH}}{\text{NAD}^+}$ also increases and consequently the concentration of lactate (Law of Mass Action). Moreover, the lactate concentration increases to a much greater degree than does the pyruvate concentration and therefore the lactate/pyruvate ratio provides a useful indication of the extent of such an oxygen deficiency.

Huckabee (1958a and b), however, maintains that this "excess lactate" accounts for all of the incurred oxygen debt. This hypothesis has been challenged by several groups (Knuttgen, 1962; Wasserman, Burton and Van Kessel, 1965) who found that the "excess lactate" only accounts for part of the debt, the remainder being due to recovery processes other than the reconversion of lactate to pyruvate. The discrepancy is greatest at light work loads where these workers found little or no "excess lactate" produced while a measurable oxygen debt developed. At heavy work loads the "excess lactate" was more representative of the oxygen debt but still could not completely account for it. These later workers postulated that there is a threshold level of work intensity before "excess lactate" is produced (this is

more in keeping with the present findings (Fig. 4.3). Huckabee, on the other hand, claimed that "excess lactate" is produced at all work loads and that the relationship is linear.

Despite these criticisms, Huckabee's investigations have been invaluable in introducing the idea that lactate production is proportional to both pyruvate concentration and deficient oxygen supply and that pyruvate concentration must be considered when using the lactate concentration to estimate the level of hypoxia. With this in mind, the lactate/pyruvate ratio is used in this work.

In the present studies, blood lactate and pyruvate levels increase during exercise (Fig.4.3, Table 4.2), indicating increased utilisation of glucose by the muscle. The relatively anaerobic conditions during the gallop are indicated by the greatly increased lactate/pyruvate ratio. Neither lactate, pyruvate nor lactate/pyruvate ratio changes to the same degree at the canter. When the pace is restricted to a canter, therefore, the cardiovascular system can presumably carry enough oxygen to the working tissues to meet the demand. It is only when the exercise intensity is of such a magnitude that the energy requirements cannot be met by aerobic metabolism alone that lactate accumulates in the muscle. The lactate diffuses into the bloodstream and is either reconverted to glucose in the liver or oxidised further to carbon dioxide and water in

tissues with a plentiful supply of oxygen, e.g. heart, kidney etc.

One direct result of this lactate accumulation during anaerobic metabolism is the fall in blood pH and base excess characteristic of a metabolic acidosis. This is seen in both horses on which acid-base studies were performed during exercise (Table 4.2). Horse 14, in addition shows a dramatic drop in blood pCO_2 (from 38.2 mm.Hg. to 19.6 mm.Hg.) indicating that the animal is hyperventilating partly in an attempt to compensate for the metabolic acidosis by pulling the equilibrium:-

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

to the left thus raising the pH and partly to obtain more oxygen. This horse, therefore, shows signs of a metabolic acidosis with an accompanying respiratory alkalosis. (It is just such a hyperventilation which causes RQ measurements made during this type of exercise to be of questionable value).

Subsequent to completion of these studies similar findings regarding the changes in blood lactate and pyruvate levels in the horse during exercise of different intensities have been made by Takagi and Sakurai (1971). These workers, however, found decreases in blood glucose levels during exercises involving both aerobic and anaerobic metabolism in contrast to the present

studies. As stated above, however, this may merely reflect incomplete compensation by the "hepatic glucostat" for the efflux of glucose from the bloodstream into the muscles.

At one time it was thought that only carbohydrate could act as fuel for exercising muscle - reviewed by Gamill (1942). Now, however, considerable evidence exists from experiments on man (Carlson and Pernow, 1959; Friedberg, Harlan, Trout and Estes, 1960; Havel, Naimark and Borchgrevink, 1963), on the dog (Issekutz et al, 1965; Issekutz and Paul, 1966) and on the rat (Gollnick, 1967) to indicate that free fatty acids provide a large part of the energy requirements for these tissues. Carlson et al (1965) showed that FFA are used as a fuel by horses running on a treadmill at speeds up to 5 metres/sec. (analogous with a trot or canter in the present studies). The present studies show that FFA are equally important as a fuel in the galloping horse.

Lipid mobilisation from adipose tissue is one of the important effects of exercise. In the present studies lipids were mobilised to a similar extent whether the animal was cantering or galloping. This is indicated by similar increases in blood glycerol levels. Correspondingly, FFA levels increase during cantering but decrease when the intensity of the exercise increases to maximum effort i.e. to a gallop. That this decrease does not appear to be due to decreased mobilisation of lipids is

indicated by the accompanying increase in glycerol levels. Lactate is known to depress lipid mobilisation in man (Cobb and Johnson, 1963) and dog (Issekutz and Miller, 1962) and, as seen in Table 4.2, lactate levels are at their highest during galloping. In this case, however, the decrease in FFA seems to be due not to decreased mobilisation from adipose tissue but to increased uptake and utilisation by the working tissues since blood glycerol levels are elevated (although possibly to a lesser extent than if lactate levels were normal).

FFA cannot be metabolised by an anaerobic pathway in the same manner as glucose. The major product of fatty acid oxidation is acetyl CoA which, under normal conditions, is then oxidised to carbon dioxide and water via the tricarboxylic acid cycle (Fig. 4.6). Under anaerobic conditions the entry of acetyl CoA into the cycle will be depressed (because of the decreased activity of the cycle) and acetyl CoA and acetoacetyl CoA will accumulate.

It should be noted, however, that even under "anaerobic conditions" the oxygen consumption of skeletal muscle may be increased 100-fold (Ganong, 1965). This is made possible both by an increased blood flow through the tissues as a result of vasodilation in muscle with simultaneous vasoconstriction in other organs and by local chemical changes which increase the dissociation of oxyhaemoglobin allowing an increase in the A-V oxygen difference.

It appears reasonable, therefore, that FFA can be metabolised in the exercising muscle, using some of this available oxygen, at the same time that lactate is being produced from glucose by "anaerobic means". This would account for part of the increased efflux of FFA from the bloodstream during galloping. An increased utilisation by cardiac muscle is also likely since this tissue is relatively aerobic at all times. The remainder could be accounted for simply by increased uptake of FFA by muscles as a result of the greatly increased blood flow through these tissues. When the exercise ceases and the blood flow returns to normal, the FFA uptake by the muscles is exceeded by the efflux from adipose tissue and the circulating levels of FFA rise above resting levels as seen in Fig. 4.4. This increase in FFA levels after exercise has been observed in the human (Friedberg et al, 1960) and by Carlson et al, (1965) in the trotting horse.

The results above indicate that the other product of lipolysis i.e. glycerol does not appear to be of great importance as a fuel during exercise in the horse.

In the present studies, training is seen to result in smaller changes in blood lactate levels and lactate/pyruvate ratio (Fig. 4.5) suggesting either that the trained horse produces less lactate or that it disposes of it more rapidly. This latter could be due to an increased rate of reconversion of lactate to

glucose in the liver. More likely, however, is the decreased production of lactate as a result of a greater capacity for aerobic exercise with less dependence on the anaerobic glycolytic pathway. This stems from an increase in the efficiency of the cardiovascular system in supplying oxygen to the working tissues since the change in lactate/pyruvate ratio is also reduced with training. Such an increase in aerobic capacity would also allow a greater contribution from FFA oxidation to the energy supply of muscle. Increased use of FFA by trained as opposed to untrained subjects has been observed in man (Cobb and Johnson, 1963) and dogs (Issekutz et al, 1965).

One of the most significant features of these studies on blood metabolites is the finding that galloping, in contrast to cantering, results in a dramatic increase in blood lactate levels and lactate/pyruvate ratio indicating a degree of hypoxia in muscle at this pace. It is presumed that the serum enzyme increases discussed in Section II arise as a result of an increase in cell membrane permeability brought about by some stimulus arising from exercise. In the past increases in blood lactate, decrease in pH, hypoxia etc. (Zierler, 1956-8; Highman and Altland, 1960) have been suggested as possible stimuli for such membrane permeability changes.

In the present studies, however, there is little change

in any of these parameters during cantering but significant increases in serum enzyme levels are observed at this pace (Table 2.8). Similarly, although very high levels of lactate, lactate/pyruvate ratio etc. are found during galloping, the increases in serum enzymes at this pace are no greater than at the canter. These findings, therefore, suggest that neither lactate accumulation nor decrease in pH is responsible for the alterations in cell membrane permeability resulting in the efflux of cellular enzymes into the serum during exercise. More important - these findings also suggest that tissue hypoxia is not involved.

Experimentally produced hypoxia in dogs (Highman and Altland, 1960) has been shown to result in increases in serum levels of GOT, LDH, glutamic pyruvic transaminase (GPT) and alkaline phosphatase. The fact that GOT is released by this stimulus supports the idea that the mechanism is different from that involved during exercise. In fact, the effects of hypoxia resemble those found in pathologic states where the membrane permeability changes appear to be less selective and GOT is released.

There is, however, the possibility that the degree of tissue hypoxia produced in the present studies by galloping is insufficient to produce the effect seen following whole body hypoxia in the dog experiments of Highman and Altland (1960).

The increase in serum GOT in these experiments on the dog may be due to release from tissues other than muscle i.e. the GOT and GPT may be released from a hypoxic liver since this tissue contains the highest concentrations of GPT found in the dog (Cornelius, Bishop, Switzer and Rhode, 1959).

The hypothesis that hypoxia is not the primary cause of enzyme release during exercise is supported by the recent observations of Loegering and Critz (1971). This group investigated the effects of electrical stimulation of hindleg muscle in the intact dog. Despite similar degrees of hypoxia (indicated by similar arterio-venous pO_2 differences), when muscles were stimulated at 5 pulses/sec. and at 10 pulses/sec., only the latter resulted in increased serum enzyme levels. Similarly, while administration of phenoxybenzamine (an adrenergic blocking agent) was found to decrease the serum enzyme rise following hypoxia in dogs (Highman and Altland, 1960), dibenamine, a drug with similar blocking activity, did not reduce the serum enzyme response to exercise in the rat (Garbus et al, 1964).

SECTION V

Studies on the Biochemical Effects of Catecholamine Administration in the Horse

INTRODUCTION

The release of catecholamines into the bloodstream is one of the primary effects of exercise (Vendsalu, 1960). Catecholamine administration in rats and in dogs has been shown to result in elevated levels of some serum enzymes (Garbus, Highman and Altland, 1967), suggesting perhaps that catecholamine release during exercise mediates the serum enzyme response to exercise. With this in mind, an attempt was made here to simulate the effects of exercise, namely the increase in serum enzymes etc. by the administration of catecholamines to the resting horse.

Both adrenaline and noradrenaline are normally secreted by the adrenal medulla in response to exercise. Adrenaline is known to stimulate both α and β adrenergic receptors (Ahlquist, 1948) while noradrenaline stimulates mainly α-receptors (Goodman and Gilman, 1968). In order to shed some light on the relative importance of these two hormones in mediating metabolic responses during exercise in the horse, the experiments described here included the use of various catecholamines and blocking drugs - adrenaline, noradrenaline, isoprenaline which stimulates only β-receptors (Goodman and Gilman, 1968) and propanolol, a drug which selectively blocks β-receptors (Sutherland, 1970).

MATERIALS AND METHODS

Materials

Drugs

Adrenaline tartrate injection B.P. (Evans Medical Ltd., Speke, Liverpool).

Isoprenaline sulphate B.P. (Evans Medical Ltd.).

Noradrenaline tartrate (Winthrop Laboratories, Surbiton-upon-Thames, Surrey).

Propanolol hydrochloride (I.C.I. Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire).

Chemicals

The chemicals used in this section have been listed elsewhere (Sections I, III and IV). The following additional reagents were used:

Sodium chloride, Analar (BDH Chemicals Ltd.).

Sodium metabisulphite (May and Baker Ltd., Dagenham, England).

(+) Tartaric acid, Analar (BDH Chemicals Ltd.).

Water for Injection, B.P. (Evans Medical Ltd.).

Methods

Preparation and administration of drugs (routes and doses)

In each of the experiments described, drugs were administered to horses restrained in loose boxes in a "fasting" state i.e. with access only to hay, as described earlier (page 147)

Adrenaline - Adrenaline tartrate was administered intramuscularly (i.m.) at a concentration of 10 µg/Kg body weight. This route was chosen because the drug is continuously released from the injection site into the circulation over a period comparable with that in exercise. Since the pH of this injection was found to be 3.2-3.6 there was a possibility of local muscle damage at the site of the injection with the resulting release of cellular enzymes into the circulation. To assess this a control injection was prepared at the same pH and containing the same constituents (with the exception of adrenaline) as the adrenaline injection.

Adrenaline injection (B.P.)		Control Injection	
Adrenaline acid tartrate	0.18 g	Tartaric acid	0.08 g
Sodium metabisulphite	0.1 g	Sodium metabisulphite	0.1 g
Sodium chloride	0.8 g	Sodium chloride	0.8 g
Water for Injection to	100 ml.	Water for Injection to	100 ml.
pH 3.2-3.6		pH 2.9-3.0	
Sterilised by autoclaving		Sterilised by autoclaving	

4-5 ml. of this control injection were administered i.m., i.e. approximately the same volume as the adrenaline injection.

Propanolol - A solution of propanolol was prepared by dissolving propanolol hydrochloride in Water for Injection to give a concentration of 10 mg. propanolol base/ml. The solution was then sterilised by filtration using the Millipore Swinnex Filter System (Millipore U.K. Ltd., London). In blocking experiments, when adrenaline and propanolol were administered to the same animal, the propanolol (0.2 mg/Kg) was given intravenously (i.v.) 10 minutes before the i.m. administration of adrenaline.

Isoprenaline - An isoprenaline injection was prepared by dissolving isoprenaline sulphate in Water for Injection to give a concentration of 1 mg. isoprenaline base/ml. and sterilising by filtration. Because of its instability this solution was made up immediately prior to use. Since isoprenaline is known to produce a prolonged, severe tachycardia (Goodman and Gilman, 1968) it was administered i.m. at a concentration of 5 µg/Kg - half the dose used for adrenaline.

Noradrenaline - The commercially available noradrenaline tartrate solution was diluted with sterile physiological saline to give a noradrenaline concentration of 1 mg/60 ml. This solution was then administered by slow i.v. infusion (8 ml/min.) over a period of 25-30 minutes to give a dose of 10 µg. noradrenaline/Kg. This

route was chosen because of the vasoconstrictor effect of noradrenaline which could restrict absorption from an i.m. injection site.

During administration of noradrenaline, isoprenaline and adrenaline + propanolol, heart rates and electrocardiograms were monitored continuously by radiotelemetry (Aitken and Sanford, in press) to detect any abnormalities.

Measurement of serum enzymes and isoenzymes

Serum GOT, LDH, ALD and CK levels were determined as described previously (Section I). Serum LDH and CK isoenzymes were determined as described in Section III.

Measurement of blood metabolite concentrations

Blood lactate, pyruvate, glucose, free fatty acid and glycerol levels were estimated as described in the previous section (IV).

RESULTS

(1) Effect of catecholamine administration on serum levels of GOT, LDH, ALD and CK

Adrenaline was administered to 4 horses as described. Blood samples were taken before, 30 minutes after injection and thereafter at intervals for 48 hours. Fig. 5.1 shows the effect on serum levels of GOT, LDH, ALD and CK for one horse. Greatly increased levels of CK were observed following adrenaline administration with peak levels around 24 hours after injection. Although on a smaller scale, significant increases in LDH and ALD levels were also found with peak levels around 24-48 hours after injection. The effect on serum GOT was generally similar to that seen following exercise i.e. a small increase within the daily variation.

These increases were not seen when the control injection was administered, indicating that the increases were not due to local tissue damage at the site of the injection.

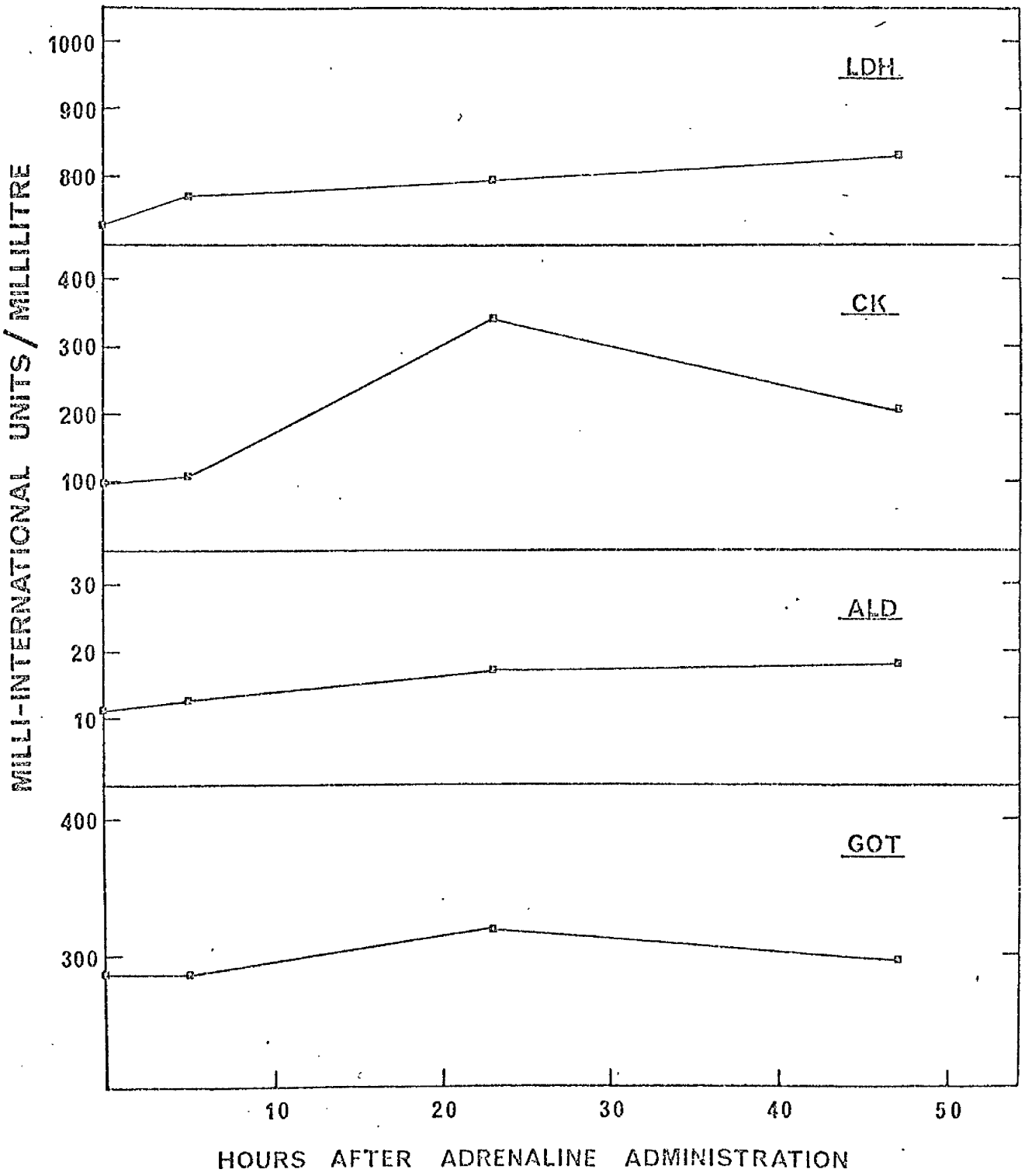
Premedication with propanolol in 2 horses did not abolish this enzymatic response to adrenaline (if anything, the increases were slightly greater - Table 5.1). It will be seen that neither noradrenaline nor isoprenaline administration produced increased levels of serum CK etc.

TABLE 5.1. EFFECT OF CATECHOLAMINE ADMINISTRATION ON SERUM LEVELS OF GOT, LDH, ALD AND CK IN THE HORSE

Horse No.	Enzyme	Increase in Enzyme Activity									
		(Adrenaline)		(Placebo)		(Adrenaline + Propanolol)		(Noradrenaline)		(Isoprenaline)	
		mIU/ml	% Increase	mIU/ml	% Increase	mIU/ml	% Increase	mIU/ml	% Increase	mIU/ml	% Increase
2	GOT	32	11			19	6			16	7
	LDH	63	10			47	9			-	-
	ALD	-	-			3.2	33			-	-
	CK	189	159			244	230				
3	GOT	46	13	26	8			11	4	31	9
	LDH	162	28	-	-			57	8	-	-
	ALD	3.4	24	-	-			-	-	-	-
	CK	489	202	14	31			6	8	-	-
6	GOT	20	10								
	LDH	99	26								
	ALD	4.4	58								
	CK	66	90								
14	GOT	9	3	16	6	60	9	-	-	4	1
	LDH	101	14	-	-	225	44	-	-	-	-
	ALD	6.4	55	-	-	7.7	103	-	-	-	-
	CK	242	247	13	20	319	385	6	8	-	-

- Indicates no increase.

FIG.5.1. EFFECT OF ADRENALINE ADMINISTRATION ON SERUM LEVELS OF GOT, LDH, ALD AND CK IN THE HORSE



(2) Effect of adrenaline administration on LDH and CK isoenzymes in horse serum

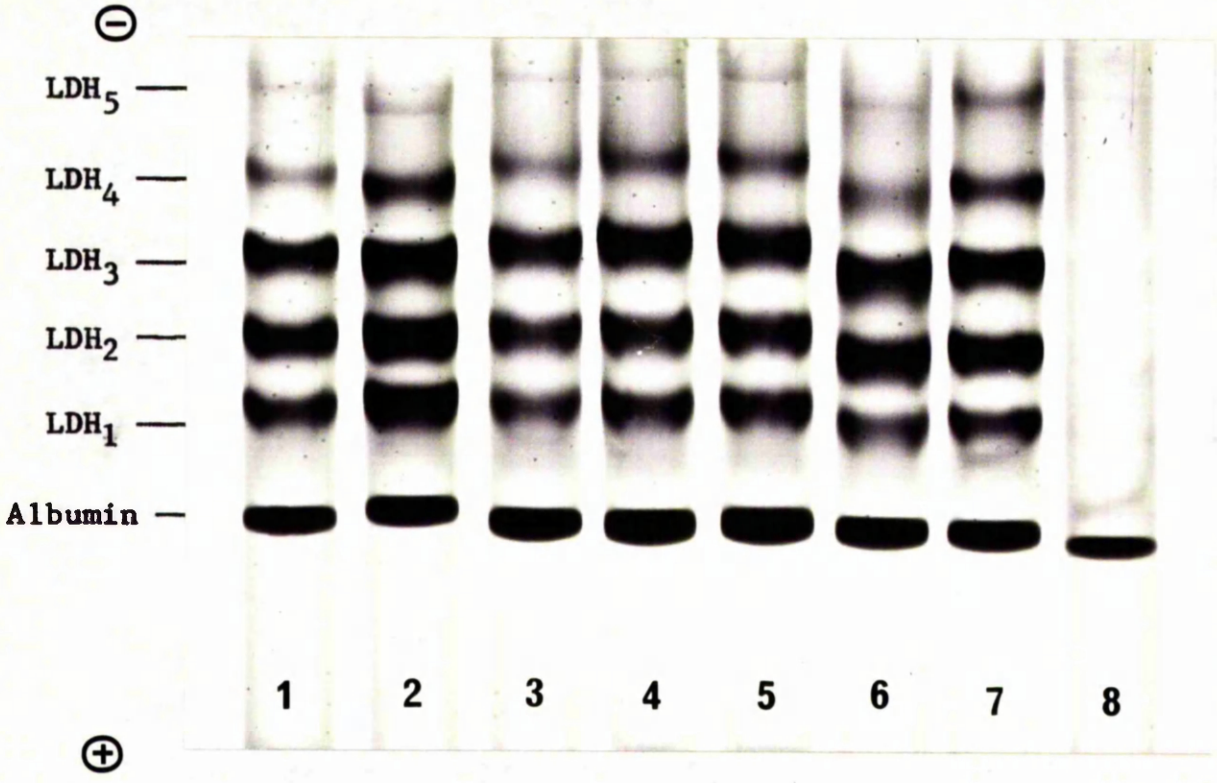
Blood samples taken before and 48 hours after adrenaline administration were assayed for LDH isoenzymes. Fig. 5.2 and Table 5.2 show the results for several horses. When the absolute activities of each isoenzyme (in mI.U/ml) are calculated, Horses 3 and 14 show an increase in all 5 isoenzymes while Horse 6 shows an increase in LDH_1, LDH_2 and LDH_3. Horse 2, on the other hand, shows an increase in LDH_1, LDH_4 and LDH_5.

The effect of adrenaline administration on the CK isoenzyme pattern of horse serum was also studied. As before (Section III) no bands of activity were seen in the control samples but the 'skeletal muscle' band, CK_{III}, became visible after adrenaline (Fig. 5.3).

(3) Metabolic responses to catecholamine administration in the horse

Blood samples taken before and 30 minutes after catecholamine administration were assayed for glucose, lactate, pyruvate, fatty acids and glycerol. The results are shown in Table 5.3. Administration of adrenaline produced a mild hyperglycaemia which was not abolished by premedication with propanolol. This effect was also produced by noradrenaline but not by isoprenaline. Lactate levels became elevated to various degrees following

FIG. 5.2. EFFECT OF ADRENALINE ON LDH ISOENZYME COMPOSITION OF HORSE SERUM

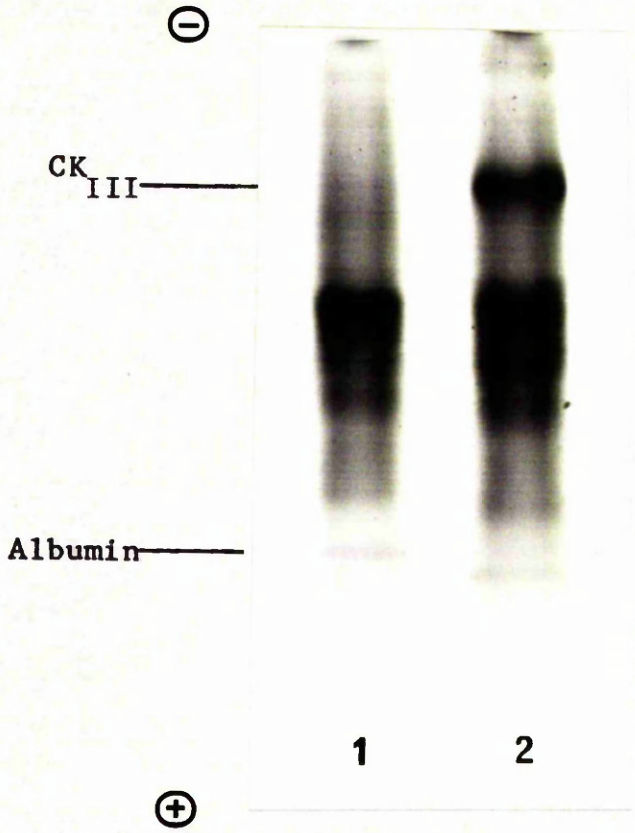


Gels contain serum from (1) Horse 14 before adrenaline
(2) Horse 14 48 hours after adrenaline
(3) Horse 6 before adrenaline
(4) Horse 6 24 hours after adrenaline
(5) Horse 6 48 hours after adrenaline
(6) Horse 2 before adrenaline
(7) Horse 2 48 hours after adrenaline
(8) blank.

TABLE 5.2. EFFECT OF ADRENALINE ADMINISTRATION ON LDH ISOENZYMES IN HORSE SERUM

Horse No.	Hours after Adrenaline	Total LDH Activity mI.U/ml.	LDH_1 %	LDH_1 Absolute Activity mI.U/ml.	LDH_2 %	LDH_2 Absolute Activity mI.U/ml.	LDH_3 %	LDH_3 Absolute Activity mI.U/ml.	LDH_4 %	LDH_4 Absolute Activity mI.U/ml.	LDH_5 %	LDH_5 Absolute Activity mI.U/ml.
2	0	458	21.5	99	26.5	121	29.5	135	15.1	69	7.4	34
	24	565	23.8	134	22.9	129	23.6	134	16.8	95	12.9	73
6	0	386	26.3	101	20.5	79	24.9	96	18.3	71	10.0	39
	24	425	29.2	124	21.7	92	26.3	112	15.5	66	7.3	31
	48	485	28.5	138	22.6	110	26.4	128	15.5	75	7.0	34
14	0	512	27.7	142	26.2	134	27.3	140	13.6	70	5.2	26
	48	737	29.4	216	24.9	184	25.1	185	15.2	112	5.4	40
3	0	582	17.2	100	23.1	135	28.6	166	18.2	106	12.9	75
	48	744	17.2	128	25.6	190	28.9	215	16.4	122	11.9	89

FIG. 5.3. EFFECT OF ADRENALINE ON CK ISOENZYME COMPOSITION OF HORSE SERUM



Gels contain (1) Serum blank

(2) Serum from Horse 3 after adrenaline.

TABLE 5.3. EFFECT OF CATECHOLAMINE ADMINISTRATION ON BLOOD METABOLITES IN THE HORSE

Horse No.	Treatment	Glucose mg%		Lactate mg%		Pyruvate mg%		Lactate/ Pyruvate Ratio		FFA mEq/l		Glycerol mg%	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
2	Adrenaline	77	93	5.2	23.1	0.37	0.89	14.1	25.9	0.17	0.45	0.40	1.36
3		84	111	6.0	9.7	0.44	0.62	13.6	15.7	0.30	0.72	0.46	1.44
6		76	99	6.3	11.0	0.45	0.63	14.0	17.4	0.28	1.10	0.48	1.68
14		79	72	4.0	6.1	0.46	0.55	8.7	11.0	0.27	1.10	0.59	2.17
2	Adrenaline + Propanolol	80	99	4.7	8.3	0.41	0.61	11.4	13.5	0.10	0.10	0.50	0.63
14		82	100	3.4	4.8	0.55	0.52	6.2	9.3	0.04	0.04	0.42	0.43
3	Noradrenaline	102	161	10.0	6.4	0.58	0.28	17.2	22.9	0.06	0.12	0.61	0.95
14		78	140	3.1	2.3	0.39	0.34	7.9	6.8	0.11	0.26	0.48	1.09
3	Isoprenaline	134	129	5.2	18.4	-	-	-	-	0.03	0.74	0.25	2.70
14		70	67	6.6	28.6	0.45	0.53	14.6	53.9	0.22	0.96	1.43	4.02

adrenaline and isoprenaline administration but not noradrenaline. Premedication with propanolol did not block this effect completely. The lactate/pyruvate ratio (which is an indication of O_2 debt) was elevated after isoprenaline and slightly elevated after adrenaline administration. Lipolysis was increased by adrenaline, isoprenaline and to a small extent by noradrenaline as shown by the increased levels of circulating free fatty acids and glycerol after administration of these drugs. Propanolol blocked this response to adrenaline.

(4) Effect of catecholamine administration on heart rate

Adrenaline, by itself and after premedication with propanolol, produced little change in heart rate. Noradrenaline decreased the rate by about 30-40% within 5-10 minutes of administration and isoprenaline increased the rate by 94-360% within 10-20 minutes of administration. Some cardiac irregularities were observed i.e. occasional dropped beats, after administration of noradrenaline and adrenaline + propanolol. (These results were obtained in conjunction with Dr. M.M. Aitken).

DISCUSSION

Previous studies on the effects of catecholamine administration on serum enzyme levels have been carried out on a number of species including the dog, rat, rabbit and guinea pig (Highman, Maling and Thompson, 1959; Garbus *et al*, 1967). The enzymes studied include GOT, glutamic pyruvic transaminase (GPT), alkaline phosphatase (AP), ALD and LDH. In each of these studies, however, very large doses of adrenaline (ranging from 0.5-5.0 mg/Kg) were administered either by i.v. infusion or subcutaneously. These doses are unrealistic and are of such a magnitude that it seems unlikely that such doses would ever be found under conditions other than experimental. In addition, when such high doses are administered, extensive tissue damage including liver damage is possible. By histological means, Highman *et al* (1959) and Garbus *et al* (1967) demonstrated fatty changes in several tissues including heart, liver, kidney and muscle. Many of the animals treated developed myocarditis and some showed signs of necrosis of liver and skeletal muscle tissue.

To avoid these complications and to obtain a more realistic increase in circulating levels of adrenaline, the dose used in the present studies is about $^{1}/_{100}$th those used previously.

As seen above, this dose is quite sufficient to produce significant metabolic and enzymatic effects.

In the above experiments, the administration of adrenaline to the resting horse resulted in elevated serum levels of LDH, ALD and CK (Fig. 5.1, Table 5.1). This suggests that the release of adrenaline by the adrenal medulla during exercise (Gray and Beetham, 1957; Vendsalu, 1960) may be at least partly responsible for the enzyme increase observed following exercise.

Several points must be considered, however. (1) The peak levels of CK etc. after adrenaline administration occur much later than after exercise; (2) the increases in CK after adrenaline are greater relative to the increases in LDH and ALD than those found after exercise; and (3) the effect of adrenaline on the serum LDH isoenzyme pattern differs from that found post-exercise.

In experiments in the dog, Highman et al (1959) found elevated serum levels of GOT, GPT and AP after administration of large doses of adrenaline and noradrenaline (0.5-1.0 mg/kg). These workers observed that the time required for cellular enzymes to appear in the serum depends on the route of administration. When adrenaline was administered by intravenous infusion, peak levels of GOT were found approximately 6 hours

after drug administration while subcutaneous injection produced a 24-hour peak with higher levels. These findings may explain the different time sequences observed in the present studies. During exercise adrenaline is released directly into the blood (analogous with the i.v. infusion) while, in the above experiments, the drug was administered intramuscularly and would, therefore, be slower in producing its effects.

Why the elevation in serum CK levels following adrenaline administration should be greater than following Exercise B (90-385% increases compared with 27-82% in Table 2.6) is less easy to understand. If it were merely a matter of higher circulating levels of catecholamines being produced in the above experiments than exist during this exercise, the increases in LDH and ALD should also be greater. Instead the LDH and ALD increases observed are of the same order of magnitude as those found post-exercise. The increases in CK, therefore, are out of proportion to those of LDH and ALD if the enzymatic response to exercise is to be explained by the effects of catecholamines alone. It appears, therefore, that some other stimulus is also involved. Garbus, Highman and Altland (1964) reached a similar conclusion during investigations on the exercised rat.

The results above suggest that adrenaline produces a more selective change in muscle cell membrane permeability, making

it easier for CK which is the smallest of these enzymes, to escape.

The results, however, also show that LDH does escape and from several tissues as indicated by the increases in all 5 isoenzymes (Table 5.2). This observation was also made by Garbus et al (1967) who found increases in all 5 isoenzymes of LDH in the serum of the rat, rabbit, dog and guinea pig following administration of adrenaline. This suggests a general increase in cell membrane permeability i.e. several tissues are affected. Following exercise (Table 3.7) the main increases are in LDH_4 and LDH_5 with smaller increases in LDH_1 and LDH_2, These were interpreted as indicating the involvement of skeletal muscle and liver but not of heart because of different rates of release and elimination of LDH_1 and LDH_5. Following adrenaline administration the relative increase in LDH_1 and LDH_2 is greater, suggesting that, in this case, heart, kidney or erythrocytes may be involved. Of these, erythrocytes are the most likely source since neither CK_I (from horse kidney) nor CK_{II} (from heart, mainly) are detected post-adrenaline. As discussed earlier, however, failure to detect CK_{II} does not rule out heart involvement since this band is the minor component of heart CK activity and the method is not sufficiently sensitive at low CK activities. Garbus et al (1967) observed that the increase in LDH_1 and the appearance of a haemoglobinaemia were blocked together by Dibenamine (an α-blocking drug), suggesting that the increase in this band was

coming from erythrocytes.

These observations, therefore, lead to the conclusion that the increase in circulating catecholamines found during exercise, although probably involved, can only be partly responsible for the alteration in cell membrane permeability which allows cellular enzymes to "leak" into the bloodstream during exercise. Some other stimulus arising from exercise must also contribute to this effect but discussion of this will be postponed until later.

In the experiments on other species, the serum enzyme elevations following adrenaline could be prevented to some degree by the prior administration of either Dibenamine or phenoxybenzamine, drugs which selectively block α-receptors. The increases in serum GOT, GPT, LDH and ALD but not alkaline phosphatase were blocked by these drugs. This together with the fact that the increases could be produced by noradrenaline instead of adrenaline indicates that the effect is mediated mainly through α-type receptors in these species.

In the present studies, the finding that the enzymatic response to adrenaline is not blocked by propanolol or produced by isoprenaline (Table 5.1) suggests that an α-type receptor should be involved in the horse also. No response was, however, obtained after noradrenaline administration. This leads to the

conclusion either that the response is mediated by some type of receptor other than α or β or that the noradrenaline dose was insufficient. Noradrenaline administration, on the other hand, resulted in a hyperglycaemia in addition to producing a reflex bradycardia in each animal treated. Therefore, unless the dose required to affect the cell membrane is very much greater than that mediating metabolic and physiological responses, the noradrenaline should be effective. Further studies would be necessary to determine if the failure to mediate an enzymatic response with noradrenaline is due to insufficient dosage or to the response being mediated by some effect of adrenaline other than that on α- and β-receptors.

(Of particular interest here is the finding that isoprenaline administration has no effect on serum enzyme levels. During each of the isoprenaline experiments a very pronounced tachycardia was observed with heart rates reaching 168 and 130 beats/minute respectively. The heart rates remained elevated i.e. above 100 beats/minute for 1-2 hours after injection. In these cases, therefore, the demand on the heart muscle was probably as great as that made in any of the exercises described and still no increase in serum enzymes was observed).

The results above also provide information on the mechanisms by which metabolic responses to catecholamines are

mediated in the horse.

The hyperglycaemic effect of adrenaline results from liver glycogenolysis rather than from muscle glycogenolysis since muscle does not contain the glucose-6-phosphatase necessary for glucose formation from glucose-6-phosphate. (Adrenaline, however, may cause the production of lactate in muscle and this lactate can be reabsorbed from the circulation by the liver and converted to glucose again). It should be noted that in the present studies, although the animals are designated "fasting", the liver glycogen stores will still be substantial despite the withdrawal of concentrates since the horses have access to hay which is fermented continually in the caecum of this species. From the above results the indications are that liver glycogenolysis in the horse is mediated by an α-type receptor since an increase in blood glucose is produced by adrenaline and noradrenaline but not by isoprenaline (Table 5.3). This resembles the situation in the rat (Fleming and Kenny, 1964) and in man (Antonis, Clark, Hodge, Molony and Pilkington, 1967) where liver glycogenolysis is also thought to be mediated by α-type receptors. In the dog, on the other hand, liver glycogenolysis appears to be mediated by β-type receptors (Mayer, Moran and Fain, 1961) in the same manner as muscle glycogenolysis. Muscle glycogenolysis is indicated by lactate production. Therefore, muscle glycogenolysis in the horse

appears to be governed by β-type receptors since lactate levels are increased following isoprenaline and adrenaline but not noradrenaline administration. Experiments in the other species - dog, rat and man (Mayer et al, 1961; Fleming and Kenny, 1964; Antonis et al, 1967) reveal that in these species muscle glycogenolysis is also mediated by β-receptors.

One of the other important effects of adrenaline is the mobilisation of fat from adipose tissue to working tissues e.g. skeletal muscle, heart muscle etc. Lipolysis is indicated by free fatty acid and glycerol production and appears to be mediated by β-receptors in the horse as in other species (Pilkington, Lowe, Robinson and Titterington, 1962; Cronin, 1967). After noradrenaline administration, however, slight lipolysis is observed (Table 5.3). This indicates either that noradrenaline has some activity on β-type receptors or that lipolysis in the horse is mediated to a small extent by α-receptors. Since, however, the lipid-mobilising effect of adrenaline is blocked by the administration of propanolol the effect of noradrenaline is probably due to its slight β-activity.

Carlson, Fröberg and Persson (1965) found increased levels of FFA, glycerol and lactate after adrenaline administration with decreased blood levels of glucose, this latter being in contrast to the present findings.

The question arises which of the two naturally occurring catecholamines, adrenaline or noradrenaline, is responsible for mediating these metabolic effects during exercise. Both hormones are secreted by the adrenal medulla in response to exercise. In addition, noradrenaline is liberated by adrenergic nerve endings and this source is said to account for most of the circulating noradrenaline under normal conditions (Euler, 1946). The circulating levels of adrenaline and noradrenaline in the horse during exercise do not, however, appear to have been investigated. The only conclusions that can be drawn, therefore, are that either catecholamine could be responsible for liver glycogenolysis and possibly lipolysis while only adrenaline could mediate muscle glycogenolysis.

Recent work in the rat (Gollnick, Soule, Taylor, Williams and Ianuzzo, 1970) has, however, shown that, in this species at least, catecholamines are not essential for lipolysis etc. and that metabolic effects can be mediated by other stimuli. By the use of various treatments including adrenalectomy, hypophysectomy, ganglionic blockade and β-adrenergic blockade, these workers observed that, in treated rats made to exercise, glycogenolysis still proceeded even in the absence of normal hormonal control, indicating that glycogenolysis can be stimulated by local metabolites or oxygen supply. To abolish

lipolysis in the exercising rat required hypophysectomy plus α-adrenergic blockade. These findings, therefore, indicate that metabolic responses to exercise can be mediated, in the rat at least, in the absence of catecholamines. Whether such a situation exists in the horse remains to be seen.

In conclusion, therefore, the administration of adrenaline to the resting horse results in increases in the serum levels of CK, LDH and ALD. These increases, however, are not identical with those obtained after exercise and some other stimulus, in addition to catecholamine secretion, appears to be involved during exercise. The results are also consistent with the hypothesis that the receptors mediating muscle glycogenolysis and lipolysis in the horse are predominantly of the β-type while the receptors mediating liver glycogenolysis are of the α-type.

GENERAL DISCUSSION

The preparation of a horse to perform in a particular event can be accomplished in many different ways. Racehorses are expected to run over distances ranging from 5 furlongs to over 4 miles and the training regime varies accordingly. Each trainer employs his own methods of feeding and training. There is no universally accepted training regime and assessment of the fitness of an animal is highly subjective and may, in some cases, be based on its performance in a preliminary race.

In the human field, much work has been done in assessing fitness and in studying the biochemical changes associated with the training process. In particular, since success in athletic pursuits depends largely on efficient functioning of muscular tissue, the release of enzymes from this tissue has been studied extensively.

In the present studies, the release of intracellular enzymes from horse muscle has been examined under various conditions in an attempt to correlate this phenomenon with the fitness of the animal. It has been shown that exercise in the horse results in increases in the serum levels of CK, LDH and ALD and that these increases are greater in the untrained animal. This finding indicates that the measurement of serum enzyme levels, particularly CK (because of its specificity for muscle), might be of some value as an index of training in this species.

In the past, physiological tests based on heart rate and respiratory rate recovery times have been used to assess fitness in man (Winton and Bayliss, 1955) and in the horse (Banister and Purvis, 1968). There has, however, been some disagreement over the practical applicability and value of these tests since, in the horse, the heart rate is affected by many factors other than exercise (Witherington, 1971). Auditory and visual stimuli may produce increases in heart rate of up to 200 per cent (Aitken and Sanford, in press) and the procedure of auscultation also influences the heart rate. Serum enzyme levels are less labile and are measured several hours after exercise and should be less subject to these influences.

Whether a test based on the increase in the concentration of serum CK after exercise would lead to similar conclusions to tests based on physiological changes, has not been fully studied. One animal, however, which was assessed as fit on the basis of heart rate recovery times after being lunged at the canter for 20 minutes daily, when subjected to one of the exercise programmes described above (Exercise B), showed significant increases in serum LDH, ALD and CK levels suggesting that, as far as this exercise was concerned, the animal was not fully trained. When Exercise B was repeated at weekly intervals, the magnitude of these increases was progressively reduced.

This finding may reflect the greater work done during

Exercise B which was of longer duration than the lunge test and involved carrying a rider. It also emphasises the importance of using a training programme adapted to the type of event in which the animal is to participate.

The problem of diet during training is of interest. In the human field, athletes tend to have a favourite diet which they adopt prior to a race. Many athletes, particularly the Swedish cross-country skiers (Astrand, 1967), exercise to exhaustion 1-2 weeks before an event to deplete muscle glycogen stores, maintain a low-carbohydrate diet until a few days before the event and then switch to a high-carbohydrate diet for the remaining period. This increases the glycogen storing capacity of muscle thereby improving the athlete's capacity for prolonged, heavy exercise.

Horse trainers appear to use methods of feeding a horse in preparation for a race, which are based on experience rather than on any scientific evidence. The digestive system of the horse provides a considerable amount of short chain volatile fatty acids some of which are stored as lipids. In this species, the importance of lipids i.e. free fatty acids as a fuel during exercise may be greater than in the human.

It should be noted that since exercise did not cause the release of all of the intracellular enzymes into the circulation, it was postulated that a temporary, selective alteration in cell membrane permeability allowing the release of only some enzymes was involved. The mechanism by which this

permeability change is brought about is still obscure. Tissue hypoxia was considered unlikely to contribute to the increase in membrane permeability in the present studies since the release of cellular enzymes was noted in the absence of a detectable hypoxia. It was also shown that i.m. injection of adrenaline into the resting horse resulted in an increase in serum levels of CK, LDH and ALD. The attractive feature of the permeability changes caused by adrenaline is that there is no efflux of GOT, which is in keeping with the findings in exercise. On the other hand, adrenaline seems to result in a more selective permeability change than that occurring in exercise since CK seems to be released more easily. Catecholamine secretion during exercise was therefore assumed to be only partly responsible for the permeability change. Despite the vast amount of research which has been carried out on the physiological and biochemical actions of adrenaline, its effects at membrane level are not fully understood.

The magnitude of the increases in serum enzyme levels appears to depend more on the duration of the exercise than on the intensity with greater increases being observed for Exercise A (duration - approximately 80 minutes) and Exercise C (duration - approximately 40-45 minutes) than for Exercise B (duration - approximately 30-35 minutes). These findings suggest that the

length of time for which the animal is exposed to some stimulus arising from exercise influences the magnitude of the membrane changes. This idea is supported by an observation of Highman et al (1959) who noted that during adrenaline infusions in dogs, one animal showed much greater increases in serum enzyme levels when it received 1 mg/Kg adrenaline over a period of 326 minutes than when it received the same dose at a faster rate over 83 minutes.

It would be interesting to compare the circulating levels of catecholamines in the horse at the canter and the gallop. Such studies do not appear to have been made up to the present. It would, however, be necessary to avoid the possibility of adrenaline release as a result of the sampling process. Horses, particularly thoroughbred horses, need to be trained to sampling although the animals used in the present study appeared to show no agitation on repeated sampling.

Neither tissue hypoxia nor catecholamine secretion appeared to be the stimulus for the increased cell membrane permeability during exercise. Little is known of the nature of the cell membrane and the mechanisms by which it controls the intracellular environment and passage of materials into and out of the cell. Although information is available on processes such as glucose transport and the "sodium pump" the mechanism by which large molecules such as proteins cross the membrane

is still unknown.

Some information can, perhaps, be obtained from a phenomenon observed in certain breeds of pig. Pietrain pigs, in particular, have been shown to be "stress susceptible" i.e. in conditions of stress, some animals develop an acute metabolic acidosis which often proves fatal. This phenomenon occurs in Pietrain sows during parturition, boars during service, during blood sampling and under anaesthesia and these pigs show elevated serum levels of CK and ALD (Allen, Berrett, Harding and Patterson, 1970; Patterson and Allen, 1972). Similarly, Hyldgaard-Jensen (1967, 1971) observed increased plasma levels of LDH, particularly LDH_5, in Danish Landrace pigs during transportation and slaughter. Each of these investigators connected the elevated serum enzyme activity with an increased anaerobic metabolism in porcine muscle indicated by the accumulation of lactic acid in the tissues. The condition, particularly under halothane anaesthesia, appears to be accompanied by increased heat production. A similar condition known as malignant hyperthermia has been observed in man (Britt and Gordon, 1969; Davies, Packer, Titel and Whitmarsh, 1969) and susceptible individuals also show elevated resting CK levels indicating a greater resting membrane permeability.

If the increased cell membrane permeability is associated with an increased anaerobic metabolism, as these investigators suggest, a shortage of available intracellular ATP might be implicated. In fact, Harrison, Saunders, Biebuyck, Hickman, Dent, Weaver and Terblanche (1969) noted a drop in the ATP content of muscle in affected pigs. In addition, Patterson and Allen (1972) suggest that the ATP concentration of muscle in stress-susceptible pigs may be lowered by a highly active ATPase.

The functioning of active transport processes such as the "sodium pump" are essential for the maintenance of a healthy, intact cell. The "sodium pump" is now known to have an absolute requirement for ATP. Should the ATP concentration become depleted either as a result of increased usage for other purposes e.g. muscle contraction or as a result of decreased production, the "sodium pump" may fail and the membrane be unable to function adequately. Patterson and Allen (1972) suggested that the increased utilisation of anaerobic pathways for glucose metabolism in porcine muscle leads to a decrease in ATP production by the tricarboxylic acid cycle and by oxidative phosphorylation. Halothane has been postulated to act by uncoupling oxidative phosphorylation from the electron transport system (Wilson, Nichols, Dent and Allen, 1966; Wang, Moffitt and Rosevear, 1969) - a process which would also result in decreased ATP production.

In exercise, the dependence of muscle metabolism on anaerobic mechanisms which give a poorer yield of ATP/molecule of glucose oxidised might be expected to result in depleted ATP levels particularly since ATP is being used up rapidly for muscle contraction. On the other hand, ATP is regenerated from the phosphocreatine reserves of the cell and possibly by the action of adenylic acid kinase e.g.

$$2ADP \rightleftharpoons ATP + AMP$$

Just what the capacity of muscle is to maintain its supply of ATP during high energy demand as in exercise, is difficult to assess. In the present studies the membrane permeability changes appear to be comparable under aerobic and anaerobic conditions suggesting, if ATP depletion is the stimulus for these changes, that the depletion of ATP is similar under both these conditions.

Whether a breakdown of the membrane integrity as a result of failure of the "sodium pump" etc. would result in cell death, as in pathological states, or whether the changes would be reversible and selective enough to account for the efflux of only some intracellular enzymes into the circulation, as in exercise, is not known. Failure of the "sodium pump" should lead to the efflux of potassium out of the cell with a corresponding increase in the plasma concentration. It is of interest, however, to observe that serum enzyme increases are frequently seen without

accompanying increases in plasma potassium concentration. Pietrain pigs have normal levels of plasma potassium despite having elevated resting serum enzyme levels. In the "stressed" Pietrain pig, on the other hand, moderate increases in plasma potassium are observed. In this stressed condition, therefore, the "sodium pump" might be functioning inefficiently. No measurements of plasma potassium concentration in the horse during exercise were made in the present studies.

Several other substances have been shown to produce changes in cell membrane permeability. These include histamine and an unknown substance prepared by Wells (1969) from tissue extracts. Both these substances, when administered by i.m. injection as an emulsion in peanut oil, caused increases in serum LDH. Wells (1969) suggests that these active substances in tissue extracts might possibly be related to the peptide kinins (Schachter, 1964) although this has not been proved. It should be noted that injection of histamine causes the release of catecholamines into the circulation (Burn and Dale, 1926) and, as has been shown above, adrenaline affects the cell membrane permeability. Histamine, therefore, may produce its effect on serum LDH levels indirectly via the release of adrenaline.

Administration of prednisolone (a synthetic analogue of hydrocortisone) has been shown to reduce the increase in serum GOT following myocardial infarction (Huzino, Kimura,

Aburaya and Katunuma, 1963). Similarly administration of prednisolone to dogs prior to exercise reduced by about 50% the increase in serum CK found in untreated, exercised dogs (Wagner and Critz, 1968). Several mechanisms have been postulated to explain this effect of glucocorticoid hormones. According to Agarwal and Garby (1964) prednisolone binds to sulphydryl groups on the cell membrane, these groups being important for the maintenance of membrane integrity. Alternatively, Grollman (1965) suggests that glucocorticoids alter cell membrane permeability by inhibiting the hyaluronidase system. Further investigation into the manner in which these hormones protect the cell membrane might provide information on the present problem.

It is obvious, therefore, that the nature of the stimulus and the mechanism by which cell membrane permeability is altered during exercise remains very much an open question.

Several other questions raised by the present studies remain unanswered. How can the delay in the appearance of peak levels of serum CK, LDH etc. after exercise, be explained? This delay indicates a prolonged change in cell membrane permeability. How can a short period of exercise or the rapid infusion of adrenaline produce an effect which persists long after their effects on the cardiovascular system? One possibility is that the cell membrane takes a long time to recover from the "damage" -

this time being required for protein synthesis. If this is so, it should be possible to delay or prolong recovery even further by inhibiting protein synthesis and to note the effect on the time course of serum enzyme elevations.

The manner in which training produces its effect also remains uncertain. This may be due to decreased circulating levels of catecholamines (or other stimuli) in a trained animal. Another hypothesis is that, during training, damaged sections of the cell membrane are gradually replaced by more resistant membrane structures. This was suggested in 1964 by Garbus et al, but, to date, this line of research does not appear to have been pursued. Our findings that the resting levels of serum enzymes decreased during the training period suggest that the training probably reduces the efflux of enzymes from the cell into the serum by making the cell membrane less susceptible to "damage". Although intracellular enzyme activities during training were not measured in the present studies, experiments on the rat have shown that the concentration of ALD and succinic dehydrogenase within the muscles increases with training (Hearn and Wainio, 1956, 1957). Increased rather than decreased intracellular levels might be expected to result in increased circulating levels. This supports the idea that training makes the cell membrane more resistant to normal "wear and tear" and presumably to other stresses such as exercise. Training could increase the efficiency and speed of the recovery process by

increasing the rate of protein synthesis. This would lead to a shorter period of increased cell membrane permeability with a resulting smaller change in serum enzyme levels.

It is evident, therefore, that there is considerable scope for further research into the mechanisms of release of cellular enzymes into the circulation both during exercise and in pathological conditions in the horse and in other species.

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SUMMARY

The present study was undertaken to obtain information on some of the biochemical changes which take place in the horse during exercise and training. Of particular interest were changes in the serum levels of certain enzymes, the tissue (or tissues) of origin of these enzymes and the manner in which exercise resulted in their release into the circulation. In conjunction with these studies, the effects of exercise on various blood metabolites were investigated to determine the relative importance of carbohydrate and lipid in the energy metabolism of the exercising horse.

Since the optimal conditions for assaying enzyme activity vary with the source of the enzyme, the optimal assay conditions for horse serum GOT, LDH, ALD and CK were first determined. Using this information, the effects of controlled exercise on serum concentrations of these enzymes were studied. Serum levels of LDH, ALD and CK were found to increase following exercise and the magnitude of these increases could be correlated with the duration of the exercise and the state of training of the animal. By comparing tissue LDH and CK isoenzyme patterns with serum isoenzyme patterns before and after exercise, the tissue of origin of these enzyme increases was found to be

skeletal muscle (with some possible contribution from liver). An alteration in muscle cell membrane permeability which allows leakage of intracellular enzymes into the circulation was postulated.

Monitoring of blood levels of glucose, lactate, pyruvate, free fatty acids and glycerol before, during and after exercise indicated that both carbohydrate and lipid catabolism were increased in the horse during exercise of varying degrees. Galloping (at 11.0-13.3 metres/sec) was found to involve anaerobic metabolism to a large extent while cantering (at 5.2-6.7 metres/sec) could be maintained by aerobic metabolism alone. Training was found to increase the animal's capacity for aerobic metabolism.

Since the nature of the stimulus which causes changes in cell membrane permeability is still obscure, the possible involvement of catecholamines secreted during exercise was investigated. Although administration of adrenaline to resting horses resulted in increased serum enzyme levels, several features of this response were incompatible with the hypothesis that catecholamine secretion might be the only stimulus for enzyme release during exercise. Tissue hypoxia and lactate accumulation were discounted as possible stimuli since enzyme leakage occurred in the absence of detectable oxygen deficiency as indicated by lactate production. Several other aspects of the membrane permeability change were discussed.