THESIS

ON

FACTORS AFFECTING THE LEVEL OF NON-ESTERIFIED FATTY ACIDS IN BLOOD

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The stimulus to fat catabolism is not the quantity of fat available, but either the absence of dietary carbohydrate or the depression of carbohydrate metabolism. This leads one to think that any condition resulting in the deficiency of calories may stimulate the fat depots to release fat in the form of NEFA, in order to supply energy. Adipose tissue is probably the chief source of fat released into the blood stream. Dole, Gordon and Cherkes and others found that the level of NEFA in the plasma was profoundly affected by the nutritional state. For instance, it is raised by factors such as fasting, and diabetes with or without ketosis and lowered by glucose ingestion and amino-acid administration (for reference *see* Chapter III).

When the present work began, there were no published reports of the effect of exercise on plasma NEFA, although several have appeared more recently. The first question asked was: "Does NEFA act as an important fuel for moderate muscular exercise, such as walking and everyday activities?"

The experiment, reported in Part I in which the NEFA level during exercise was measured in a number of subjects under different conditions, leads to the conclusion that large amounts of NEFA may be mobilised and utilised during moderate exercise. The results of others published since this work began are also consistent.

The question then arose: "What factors are responsible for the mobilization of fat as NEFA?" It is now well known that the NEFA level is affected by many hormones, notably adrenaline, growth hormone and insulin etc. In Part II experiments are reported showing the effects of these hormones on NEFA levels on man and intact rats and also on the liberation of the NEFA by rat adipose tissue _in vitro_. It does not appear possible to state precisely the factor concerned in the mobilization during moderate exercise.

PART I

NEFA AS A FUEL OF MUSCLE

CHAPTER II

INTRODUCTION

PREVIOUS VIEWS REGARDING THE FUEL OF MUSCLE

CARBOHYDRATE AS THE 'PRIME FUEL' OF MUSCLE The nature of the fuel utilised by the working muscle is an old physiological problem. Many physiologists have considered that carbohydrate was the sole fuel of muscle. Cl. Bernard was of the opinion that the grape-sugar which he had discovered in the liver and in the blood might by its oxidation in the tissues take an important part in the production of heat and of mechanical energy. Seegen (1891) went much further than this, holding that muscular energy is solely obtained from the oxidation of dextrose brought to the muscles by the blood, whether glycogen was present or absent in the liver. Chaveau and Kaufmann (1887) like Seegen found a similar difference in the amount of sugar of blood in the venous and arterial blood supply of muscle. All these workers support the argument that the work is done at the expense of carbohydrate.

As long ago as 1866 Pettenkofer and Voit showed that mechanical work did not increase protein metabolism and this has since been confirmed.

In 1907, Fletcher and Hopkins revealed the relation

of lactic acid to muscular activity and this drew attention to the importance of carbohydrate in the muscle.

Subsequently, Meyerhof (1919) and A.V. Hill (1924, 1925) and others in experiments on isolated muscle preparations showed the close relation of carbohydrate breakdown and lactic acid formation with the development of muscle tension and the liberation of heat. A.V. Hill's lecture on 'Lactic acid as the keystone of Muscular Activity' (1926) helped to lead to the idea that carbohydrate was the sole fuel of muscle.

MacLeod (1928) in his book 'The Fuel of Life', has assembled the material and arguments in favour of the fat to carbohydrate transformation theory. Most biochemists became sceptical as to whether fat is normally converted to carbohydrate in the body. In this they have been supported by Rapport (1930), who showed how unsatisfactory was all the evidence in favour of the formation of carbohydrate from fat in the body.

In 1952, Lovatt Evans stated an orthodox view as follows 'Fats must often be utilized for the carrying on muscular work. For this to take place, it is probable, however, that they are first converted into carbohydrate and stored in the muscle as glycogen'. However, the classical work on carbohydrate is undoubtedly correct so far as the immediate source of fuel for a sudden burst of hard exercise is concerned. Glycogen is the one fuel immediately available in muscle

and its breakdown to lactic acid certainly provides the energy for any burst of activity. But this mechanism is probably responsible for no more than a small fraction of the energy utilised in long-continued muscular work. It will not be illogical, therefore, to suppose that carbohydrate may not be the sole fuel of muscular energy.

FAT AS A SOURCE OF ENERGY The lipids of the blood have not been studied in relation to exercise to the same extent as carbohydrate.

The biochemists often used to omit any reference to the fuel of muscle, but at one time they would have agreed with the conception of Peters and Van Slyke (1946). They observed that fat was carried from the adipose tissue to the liver, where it was converted into ketone bodies. The ketones were then available to meet the energy requirements of muscle. Modern ideas stem largely from the experiments of Stetten and Boxer (1944) with the aid of deuterium. They showed in rats on a high carbohydrate diet that only about 3% of the dietary glucose was handled as glycogen and at least 10 times as much as was used to synthesize fatty acids as to synthesize glycogen. Before that Gemill (1942) reviewed other preisotope experiments which were designed to investigate the fuel for muscular exercise. From his experiments it was

concluded that though carbohydrate was 'prime fuel', fat also can be used by muscles. This evidence was derived from the low R.Q. values found in exercising subjects or isolated muscle preparations.

The work of Andres _et al_. (1956) supports the view that fat is responsible for the production of energy. These workers measured the oxygen consumption during forearm exercise in man. They have shown that the glucose disappearance from blood cannot account for the oxygen consumption, even if the whole amount of glucose were oxidized to Co_2. On the basis of an average R.Q. of 0.80 together with other considerations, these workers have postulated that fat may be the major substrate of skeletal muscle, which supports the work of others (Geyer _et al_., 1949; Volk _et al_., 1952; Hansen and Rutter, 1952; Tepperman _et al_., 1956).

The experiments of Fritz _et al_. (1958) with isolated skeletal muscle showed that fatty acid oxidation was doubled during experimentally induced activity. It was observed that the increment of fatty acid oxidation with stimulation was greater than the percentage increase in oxygen consumption. These data demonstrate that the fuel of muscle during exercise probably includes fatty acids. The fall in R.Q. found in these experiments is also compatible with the hypothesis that fat is oxidized at a faster rate during exercise.

The data from above experiments suggest that the metabolism of fatty acids by muscles is potentially an important source of energy. This fatty acid fraction of the fat is presumably the non-esterified fatty acid, which is discussed in detail in the following chapter III.

CHAPTER III

ROLE OF NON-ESTERIFIED FATTY ACID

The fraction of the total fatty acids of blood known as non-esterified fatty acid, is metabolically important, though quantitatively small (2-5% of total blood lipids). Unesterified fatty acid (UFA) and free fatty acid (FFA) have also been used to describe the same fraction. UFA may be a true synonym, but there is always the chance of confusion with unsaturated fatty acids and NEFA is generally preferred.

The term free fatty acid is not strictly accurate, for this fraction never exists in a free form but always in loose combination with plasma albumin (Gordon _et al._, 1953; Selden and Westphal, 1955). Slightly less than one molecule of NEFA was found per molecule of albumin in normal rats, rabbits and bovine plasma and this also is the usual range in human plasma. However, one molecule of albumin can carry up to 6 molecules of NEFA (Gordon _et al._ 1953). The NEFA fraction exists in plasma primarily as anions, though at the pH of plasma about 1 per cent of the fatty acid is undissociated (Markley, 1947).

This fatty acid fraction has been recognised for about 80 years. Hoppe-Seyler (1883-84) reported the

presence in the blood of rabbits, horses and dogs of about 0.05-0.12% of the fatty acid which was not esterified. This he obtained from the serum after the analysis had been done for several times. Little interest was taken in the discovery at the time. Szent-Györgi and Tominaga (1924) obtained similar results but these were considered by other workers to be artefacts. It now appears that the NEFA concentrations found by these two workers were within the normal range.

Some years later, Kelsey and Longenecker (1941), described the recovery of NEFA by fractionation of the lipids extracted from bovine plasma. They tabulated the fatty acids found in the NEFA fraction and observed mostly palmitic, oleic and linoleic acids with small amounts of stearic, myristic and arachidonic. But their results are open to question. As the time required for processing so large a quantity of plasma (90 litres) was long, they considered that some lipid esters might have undergone hydrolysis *in vitro*, with the artefactual production of NEFA.

Cohn and his collaborators (1947), isolated NEFA which were uniformly present in the albumin fraction of human plasma. Information regarding the individual fatty acids of the human NEFA fractions is limited to one study by Dole (1957) in which by

means of silicic column chromatography, palmitic and oleic acids were identified. This experiment confirmed the report of Kelsey and Longenecker (see above). More recent and detailed fractionation with gas phase chromatography shows traces of short-chain acids starting with C_6, but no major amount is obtained until C_{16} is reached. According to Hanahan and Dittmer (1960) within the neutral lipid fraction oleic, linoleic and palmitic acids appeared to a great extent as triglyceride while stearic acid was found to be more significantly associated with cholesterol esters. These four acids comprised about 80% of NEFA fraction.

Fatty acids of short-chain length (volatile fatty acids) occur in plasma, particularly in ruminants. The methods for the estimation of these acids are such that fatty acids incorporated with glycerides or strongly bound to protein could not be recovered.

TURNOVER RATE OF NEFA According to Shreeve et al. (1956), although the plasma concentration of NEFA is low, the amount of potential energy delivered to cells by the rapid turnover of this fraction is more than double the calorie value of the glucose stream.

Table I. gives the approximate calorie values of glucose and NEFA pools along with the turnover percentage per minute (Dole, 1958).

TABLE I

	Size Cal/kg	Turnover % per minute	Outflow Cal/Kg/day
Glucose	0.84	0.7	8.5
NEFA	0.05	28.0	21.0

SOURCE OF NEFA The non-esterified acids of plasma may originate from the following sources.

1. THE HYDROLYSIS OF PLASMA TRIGLYCERIDES

The production *in vitro* of NEFA by the action of lipoprotein lipase (clearing action) on the triglycerides of chylomicrons, lipoproteins and artificial fat emulsions has been repeatedly demonstrated (Robinson and French, 1953; Shore *et al.*, 1953; Korn, 1955; Borgström and Carlson, 1957). The same process is believed to occur *in vivo* following the injection of heparin (Havel, 1957; Spitzer and Miller, 1956). It was observed that in the absence of exogenous heparin, there was a rise in NEFA levels after injection of artificial fat emulsions (Grossman *et al.*, 1955; Moeller *et al.*, 1955).

2. FROM ADIPOSE TISSUE This is the probable source of NEFA. The largest arterio-venous difference is encountered in the greater saphenous vein which drains a subcutaneous area rich in adipose tissue (Gordon, Cherkes and Gates, 1957). However, the production

of NEFA from other tissues either *in vivo* or *in vitro* is not reported. So, at least up to this time, it must be considered that endogeneous NEFA may originate solely from adipose tissue (as opposed to NEFA production from dietary fat during alimentary lipaemia).

THE EFFECT OF NUTRITIONAL STATE ON THE PLASMA NEFA

It has been found that the concentration of NEFA in the plasma was increased during fasting (Laurell, 1956), after adrenaline injection (Gordon and Cherkes, 1956; Dole, 1956), in diabetic acidosis (Laurell, 1956; Bierman *et al.*, 1957a) as well as in the 'controlled' diabetic state (Bierman *et al.*, 1957a) and after administration of growth hormone (Raben and Hollenberg, 1958).

On the other hand a decrease was observed after administration of glucose (Gordon and Cherkes, 1956; Dole, 1956) amino-acids (Gordon, 1957), glucagon (Bierman *et al.*, 1957b) and insulin (Dole, 1956).

Also, it was shown that addition of sugar or insulin in *vitro* decreases oxidation of fatty acids by liver slices (Haugaard and Stadie, 1952; Lossow and Chaikoff, 1955) or by sections of muscle (Wertheimer and Ben-Tor, 1952). Carbohydrate feeding decreases hydrolysis of triglycerides in the liver (Lossow and Chaikoff, 1955) and promotes synthesis of various lipids from acetate (Bloch, 1948; Lyon *et al.*, 1952; Medes, *et al.* 1952).

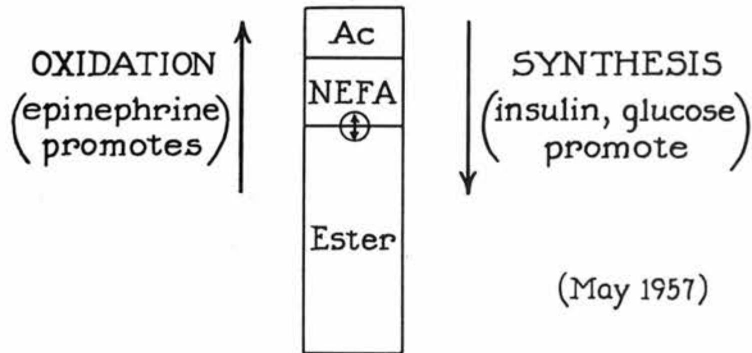

Fig. 1. Symbolic representation of the balance between acetate, NEFA and esterified lipids within cells. Although the mechanism is still obscure, it seems generally true that increased oxidation of carbohydrates shifts the balance toward synthesis (Dole, 1958, p. 194).

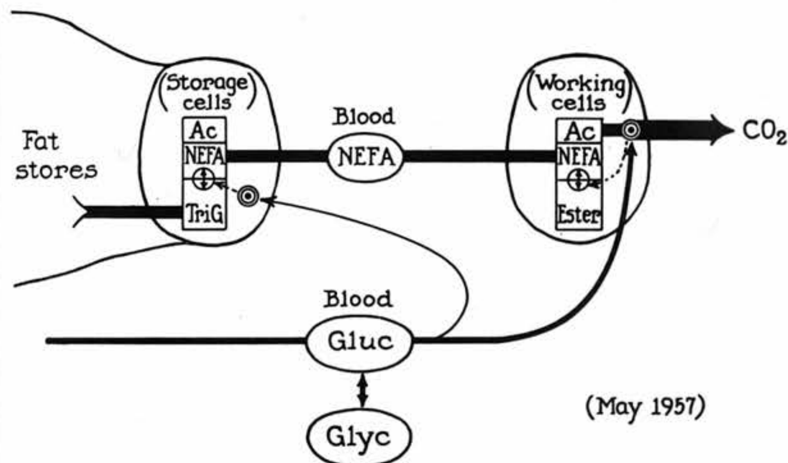

Fig. 2. Map of hypothesis. The process governing synthesis and hydrolysis of fat (Fig. 1.) is supposed to be operative in both storage and working cells. (Dole, 1958, p. 195).

All these findings have been interpreted to mean that NEFA is a major link in the transport of fat from the fat depots to the peripheral cell and the level of energy production in some way controls the movement of NEFA. It seems that utilization of carbohydrates promotes synthesis of lipids and depletion leads to oxidation. The Fig. 1 will symbolise this dynamic balance.

If the NEFA fraction is an important means of transport, as it seems to be, conversion of triglyceride to NEFA in storage cells and oxidation of NEFA to acetate in liver and other working cells must be closely correlated. The Fig. 2 may explain this (Dole, 1958).

THE PRESENT INVESTIGATION ON THE EFFECT OF EXERCISE ON THE PLASMA NEFA LEVEL

The above hypothesis implies that the outflow of NEFA from storage would vary in response to change in catabolism of fatty acids. The shift to hydrolysis of the triglyceride from the adipose tissue will probably increase the plasma level.

All the work done by previous workers, which was mentioned before, showed that calorie deficiency which was due to lack of a non-fat source, resulted in the mobilization of fat as NEFA in blood. Thus

moderate exercise in the post-absorptive state which increases calorie requirements, might be expected to act as a stimulus for catabolism of fat. With this view the present experiments were planned.

CHAPTER IV

ESTIMATION OF NON-ESTERIFIED FATTY ACIDS

PRINCIPLE Fatty acids are extracted from solution in the plasma water by a mixture of lipid solvents. This separates the fatty acids from other organic acids which are soluble in water but not in lipid solvents. This results in a two-layered or two-phase system.

EXTRACTION MIXTURE Folch, Lees and Sloane-Stanley (1954) used methanol-chloroform as an extraction mixture. Blankenhorn and Ahrens (1955), employed heptane-ethanol. Dole (1956) introduced heptane and isopropyl alcohol, finding heptane to be a more convenient solvent than chloroform. This mixture contains isopropyl alcohol, 40 parts, heptane, 10 parts and $1NH_2SO_4$, 1 part. During the present investigation it was found that with this proportion it was difficult to get a two-layered system, and different proportions of fat solvents were tried. To get the best separation of the layers two different extraction mixtures were used. Mixture A consisting of isopropyl alcohol, 30 parts, heptane, 20 parts and $1NH_2SO_4$, 1 part was used for plasma and unknown aqueous solutions. Mixture B, consisting of isopropyl alcohol, 30 parts, heptane, 10 parts and $1NH_2SO_4$ 1 part was used for standard solutions of palmitic acid in heptane. The standard palmitic acid

solution was made by dissolving 2.56 g. of pure palmitic acid in 100ml. heptane. For this purpose palmitic acid (B.D.H.) was re-crystallised from heptane. Purity was tested by the melting point of the re-crystallised material (m.p. 64).

The two extraction mixtures were used in order to get the same volume of the upper layer (5ml.) in each case even when the solution contained heptane.

In the preparation of extraction mixtures and standard solution, re-distilled isopropyl alcohol and heptane were always used.

<u>TITRATION MIXTURE (INDICATOR)</u> A stock solution of thymol blue was prepared by dissolving 100 mg. in 100 ml. of 90% alcohol. For everyday use the stock solution was diluted in the proportion of 1:10 with absolute alcohol. So, the strength of this became 0.01%. As the titrable acidity of the alcohol increases over a period of several days, freshly prepared mixture must be made just before the titration is started (Dole, 1956).

<u>ALKALI</u> 0.02N NaoH was used. It was made by diluting a stock solution of N/10 NaoH.

<u>PROCEDURE</u> Blood was collected in a syringe or vessel containing two drops of heparin ('Liquemin', Roche). The blood was transferred to a centrifuge tube within 10 min. and centrifuged at 3,000 r.p.m. for about 10-15 min. 1 ml. of plasma was transferred to a

glass-stoppered test tube and 5 ml. of extraction mixture were added to it. It was then shaken vigorously for about a minute and allowed to stand for 10 min. 2 ml. heptane and 3 ml. distilled water were added. Dole used CO_2-free distilled water. This refinement was found to be unnecessary, if either freshly distilled water or de-ionised water was used. The only precaution necessary is that water must be freshly distilled from glass and kept in a well corked flask provided with a side tube containing Calsoda to absorb atmospheric CO_2. The test tube was again shaken vigorously for 30 sec. and left for 10 min. as before.

1 ml. of distilled water and 1 ml. of standard palmitic acid were treated in the same way except the extraction mixture B was added to the latter. These serve as the 'blank' and 'standard' respectively. After standing for 10 min., the liquid in the test tube was separated into two phases. The upper 5 ml. contained the fatty acid in heptane. From this a 3 ml. aliquot was transferred to a 10 ml. conical centrifuge tube containing 1 ml. of titration mixture. This was titrated with alkali by using the micro-burette of a 'Kopp Natelson Micro-gasometer Model 600'. The burette is attached to the reservoir of this apparatus

by means of a connector, and is of 0.12 ml. capacity and calibrated in wide divisions of 1 μl. This reduces the reading error and permits estimation to 0.1 μl. The burette communicates with the micro-gasometer by means of two stop-cocks. Filling and emptying the burettes is done by means of a hand wheel attached to the base of the micro-gasometer. The whole burette can be filled up with mercury. The burette is filled up with alkali by bringing the mercury in contact with the reagent and drawing it in.

Before the titration was started the tip of the burette was wiped out by means of a filter paper. Throughout the titration nitrogen gas was bubbled through the solution from a polythene tube. This gas, from a cylinder, at first passed through alkaline pyrogallol to absorb traces of Co_2 or O_2. Nitrogen was necessary to keep the two phases together i.e. to prevent the phases from separation (titration mixture and heptane containing the sample), as well as to expel Co_2 coming from the outside atmosphere. Before the titration was started, nitrogen was bubbled through the solution for 30 sec. to expel any Co_2 present.

The end-point is the change from pink to a greenish-yellow colour. A good light is essential to note the change in colour. For this reason a

portable fluorescent lamp was kept above and just in front of the titration tube. As the end-point was approached, the gas stream was interrupted from time to time to allow examination of the indicator in the alcoholic phase.

CALCULATION For the titration of the non-esterified fatty acid .02 N NaoH was used, containing .02 M Mole/ litre or .02 μM/μl. A 3 ml. aliquot was taken out of the 5 ml. volume of the upper phase, was titrated by NaoH and the reading recorded in μl. So, the total amount of the non-esterified fatty acids in the original sample will be calculated as follows.

Total amount of NEFA in the original 1 ml. sample

$= .02 \times \frac{5}{3}$ (the amount of NaoH added in μl - the amount of NaoH in ul required by the blank)

$= .02 \times \frac{5}{3} \times$ the corrected amount of NaoH in μl.

$= .033 \times$ the corrected amount of NaoH in ul was the amount in μM/ml.

COMPARISON WITH OTHER METHODS Dole showed that other organic acids such as pyruvic, acetic and citric do not interfere with his method. He does not mention lactic acid. In the present investigation, it was found that 2% lactic acid in the solution, did not interfere. Trout *et al*. (1960) have also shown that lactic acid does not interfere with their method, which is a slight modification of Dole's.

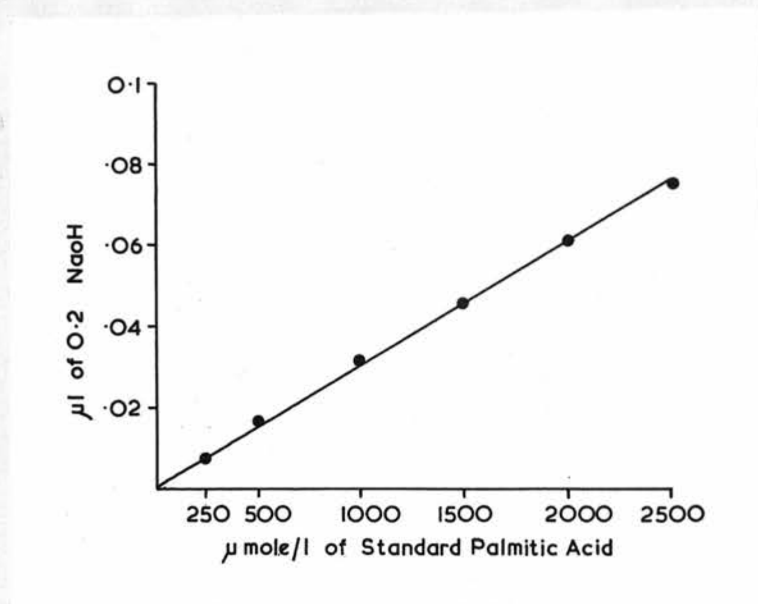

Fig. 3. See text.

There are some other methods of estimation of NEFA. Gordon, Cherkes and Gates (1957) treated the plasma after it was freeze-dried with glacial acetic acid and isoöctane. This method is time consuming as well as needing special type of apparatus. Whereas Dole's method is suitable for a clinical laboratory. A drawback is that blood sample must be analysed within 8 hours and after standing overnight on the bench or in a refrigerator there is usually a fall in the NEFA titre.

RECOVERY A known amount of the standard palmitic acid solution was added to plasma and titrated in each experiment. Table II shows the percentage recovery of palmiticacid from 100 samples. This Table shows that 90% of the results were within ± 5% and the extraction process was probably complete.

Titration of graded amounts of palmitic acid against 0.02 N NaoH in µl is shown in Fig. 3 after the blank has been subtracted from it.

TABLE II

Percentage recovery of palmitic acid added to one hundred samples

Recovery %	No. of plasmas
90-94	1
95-99	22
100-104	68
105-109	7
110+	2

CHAPTER V

EXPERIMENTAL PROCEDURE

FORM OF EXERCISE In order to observe the effect of moderate exercise on the level of non-esterified fatty acid, walking was selected as the standard activity.

Stepping, walking, and stationary cycling are all forms of exercise suited to laboratory investigation. Stepping (which usually includes stepping backwards) and cycling are forms of exercise to which not everyone is accustomed. Moreover, these forms of exericse require full co-operation of the subject to maintain a 'steady state' of work.

The concept of 'steady state' of exercise was established by the work of A.V. Hill and his colleagues (1924). Provided the rate of work does not exceed a certain limit (the 'crest-load', set by aerobic capacity), a state is reached, soon after beginning exercise, in which the O_2 uptake remains almost constant and which may be maintained for an hour or more.

During the steady state there is approximate constancy of pulmonary ventilation, arterio-venous O_2 difference, cardiac output, heart rate and other variables. Above the crest-load O_2 uptake may

TABLE III

Subject	Age	Sex	Weight (kg)	Height (cm)	NEFA m-mole / l.
R.P.	49	m	63.4	182	0.80
A.B.	32	f	73.2	160	1.26
R.J.C.	22	m	70.0	176	0.67
M.H.D.	38	m	65.5	178.8	0.69
K.B.R.	36	m	79.5	181.3	0.61
S.L.	27	f	60.0	168	0.64
P.G.	21	f	65.1	168	0.67
L.T.	23	f	63.7	177	0.67
M.B.	21	m	61.0	182	0.84
M.H.	24	m	63.5	170	0.73
M.J.H.	22	m	104.0	183	1.01
G.G.W.	21	m	63.9	178	0.88

Age, sex, weight, height and fasting plasma NEFA level of 12 healthy subjects.

become steady but the exercise can be maintained only for a short time and other variables do not reach a 'steady state'. Moreover, the use of a single standard exercise simplifies the procedure. So, in the present investigation walking is selected as the form of exercise.

SUBJECTS Twelve healthy students and research workers acted as subjects. Among them were 8 males and 4 females. Their age, height, weight and fasting level of plasma NEFA is given in Table III.

They were told not to take any food after 6 p.m. on the evening before the day of the experiment. Also they were asked to avoid carbohydrate food as far as practicable. Gordon and Cherkes (1956) formulated the hypothesis that NEFA serves during fasting as a transport of fatty acid. The present investigations were therefore carried out 14-16 hours after the last meal.

The subjects, who were not familiar with the treadmill and respiratory apparatus, came before the day of the experiment. They walked on the treadmill for some time and sample of expired air was collected. All this was done to get them accustomed to the motion of the treadmill as well as to the use of the mouth-piece of the respiratory apparatus.

All the subjects attended the laboratory at 8.30 a.m. having had no breakfast. They were warned not to drink any tea or coffee with milk and sugar before coming to the laboratory. All the subjects except one came to the laboratory without having any tea or coffee. Also, they were told not to hurry to the laboratory before the experiment.

After they arrived at the laboratory they lay down comfortably on a couch kept at the laboratory for about 20-30 minutes. The temperature of the room was kept between 65-67° C, either by extra heating during the winter or by extra ventilation during the summer.

After their rest (for 20-30 minutes), an expired air sample was collected in a Douglas bag (Douglas, 1911). Then, a blood sample was taken from the ante-cubital vein by means of a syringe to which heparin ('liquemin') was added beforehand. For, the first sample 20 ml. blood was drawn. This was centrifuged for 15 minutes and then transferred to a centrifuge tube as mentioned in Chapter IV. Before the centrifugation was done 0.1 ml. was pipetted into a centrifuge tube containing 3.8 ml. of isotonic sodium sulphate - copper sulphate solution for estimation of blood glucose.

The plasma obtained after centrifugation was transferred to a bottle which was left in the refrigerator until all the blood samples were collected.

After collection of expired air and the fasting blood, the subject started walking on the treadmill for an hour. The healthy men walked at 4 m.p.h. on the level. This was rather fast for women, who walked at 3 m.p.h.

The exercise consisted of four periods of 15 minutes each accurately timed by stopwatch. During the last 5 minutes of each 15 min. period expired air was collected and ventilation measured for determination of oxygen consumption and R.Q. After each 15 min. period the subject lay on a couch while 10 ml. of blood was taken into a heparinised syringe. This was easily accomplished within 1 min. after which the subject resumed walking up to the end of the hour's exercise.

In addition to the experiments mentioned above, seven more were done. Of these, four consisted of a 10 mile walk in 150-180 min. depending on speed. In one 25 g. of glucose was given by mouth during every 15 min. rest interval.

The sixth experiment was the heavy exercise, with a 10 kg. load on the back walking at an incline of 6% at 4 m.p.h.

The last experiment was the three consecutive runs of one mile with intervals. Each run was in 5 min. 30 sec. followed by a rest lasting 9 min. 30 sec.

METHODS

RESPIRATORY EXCHANGE A Kofranyi-Michaelis respirometer (Kofranyi and Michaelis, 1940) was used for the collection of air samples during exercise. Expired gas was analysed by the Haldane method; duplicates were required to agree within 0.02 volume per cent for both O_2 and Co_2.

CHEMICAL ANALYSIS Plasma NEFA was estimated by the method described in Chapter III. Blood glucose was estimated by the method described by King and Wooton (1956).

TABLE IV

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. Mean and standard deviations of 10 experiments Subject Healthy Age Ht. Wt.

Exercise An hour walk on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.76 ± .179	4.60 ± .811	231	0.78 ± .058
Exercise for 15 min.	0.95 ± .226	4.29 ± .839		0.83 ± .033
" " 30 "	1.08 ± .302	3.95 ± .763	1240	0.82 ± .040
" " 45 "	1.34 ± .398	3.99 ± .713	1240	0.80 ± .062
" " 60 "	1.44 ± .363	4.07 ± .804	1240	0.80 ± .045
Recovery for min.				

CHAPTER VI

RESULTS

EFFECT OF AN HOUR'S WALK ON PLASMA NEFA

Table IV summarizes the changes in the plasma NEFA, blood glucose, oxygen consumption and R.Q. in ten healthy subjects during moderate exercise. The changes in the plasma NEFA, blood glucose, oxygen consumption observed in the individual subjects are given in the appendix. The average fasting level was 0.76 m-mole per litre. This agrees with the findings of other workers. For instance, Dole (1956) found a fasting level of 0.50-0.90 mEq/l in 3 healthy males. Gordon and Cherkes (1956) found a concentration of 0.30-0.80 mEq/l in seven healthy young men. Anfinsen (1956) and Grossman *et al.* (1955) found similar values.

In the present investigation the mean value for NEFA during the exercise increased from 0.76 to 1.44 m-mole/l and for the blood glucose, fell from 4.60 to 4.07 m-mole/litre. Blood glucose is expressed as m-mole/litre instead of as mg. per 100 ml., because it seems reasonable to use comparable units. The R.Q. rose at the start and then fell slightly. The oxygen consumption changed little throughout the walk.

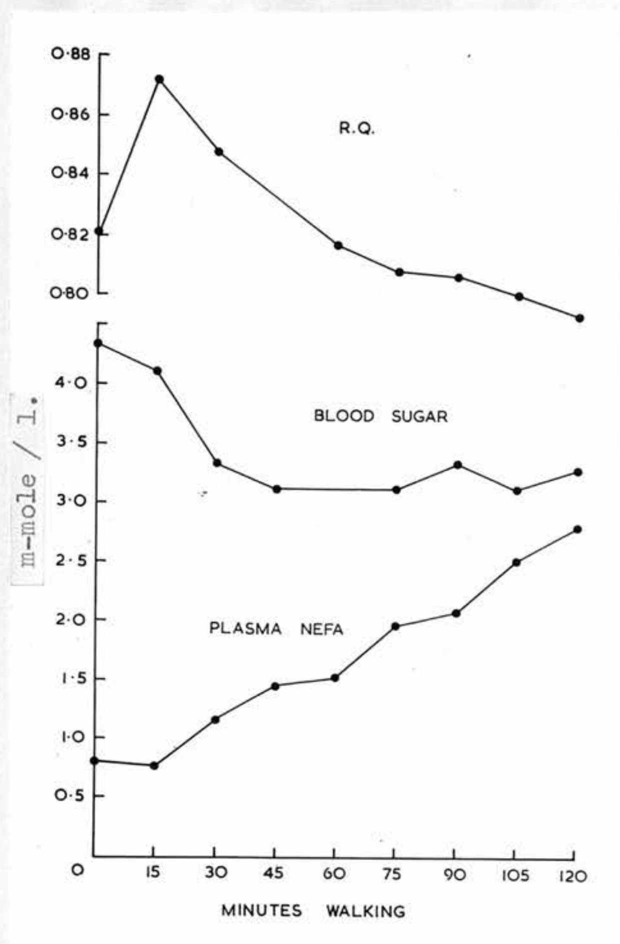

Fig. 4. The effect of walking at 4 m.p.h. in the post-absorptive state on the R.Q., blood sugar and plasma non-esterified fatty acid in a normal healthy man.

A search was made for correlations between the extent of the NEFA rise and the levels of blood sugar and the R.Q.s. in individual subjects and also the degree of obesity (as judged by the height/weight ratio). No correlations could be found, nor was any found between the extent of the rise and rates of oxygen consumption. (Results published in Quart. J. exp. Physiol. (1960) 45, 312.

ADDITIONAL OBSERVATIONS ON NEFA LEVEL IN EXERCISE

Some additional observations were recorded during walking for longer periods, after running and during heavy exercise by walking uphill with loads. Fig. 4 shows the results in one normal subject, who continued to walk at 4 m.p.h. for two hours. There was a steady rise in plasma NEFA throughout this period. The final value was 2.80 m-mole/litre. There was a gradual fall of R.Q. from 0.84 to 0.79. The blood sugar also showed a fall from 4.2 m-mole/litre to 3.28 m-mole/litre. Calculations from the respiratory exchanges and urinary nitrogen during this walk indicated that about 55 g. of fat had been mobilized and utilised during the two hours.

In two other experiments, the same subject walked for 150 minutes (Tables V and VI). In one the NEFA rose to 1.72 m-mole/litre in 60 minutes and thereafter

TABLE V

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 1 Subject R.P. Age 49 Ht. 182 cm. Wt. 63.4 kg.

Exercise Walk for 150 min. at 4 m.p.h. on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.83	-	231	0.77
Exercise for 30 min.	1.42	-	1128	0.81
" " 60 "	1.72	-	1199	0.78
" " 90 "	1.92	-	1311	0.78
" " 120 "	1.75	-	1187	0.78
" " 150 "	1.87	-	1181	0.78
Recovery for min.				

TABLE VI

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 3 Subject R.P. Age 49 Ht. 182cm. Wt. 63.4 kg.

Exercise 4 miles/hr. for 150 min. on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.95	-	230	0.85
Exercise for 30 min.	0.98	-	-	0.88
" " 60 "	1.28	-	1086	0.84
" " 90 "	2.03	-	1103	0.83
" " 120 "	2.44	-	1095	0.82
" " 150 "	2.94	-	1089	0.79
Recovery for min.				

changed little. In the other there was a steady rise which reached 2.94 m-mole/litre after 150 min. This was associated with a large fall in R.Q.

Tables VII and VIII show the rise in NEFA in two subjects during walks of 145 minutes and 180 minutes. During these two experiments blood was first drawn at 85 and 90 minutes respectively.

Table IX shows the effect of heavy exercise (walking with a load of 10 kg. at an incline of 6%) for 50 minutes, by which time the subject, a heavy man, was completely exhausted. The NEFA level was then 1.79 m-mole/litre The blood glucose did not show any consistent change but varied from 5.47 m-mole/litre to 5.11 m-mole/l. The R.Q. was high throughout the exercise, but there was a slight rise.

Table X shows the NEFA level after three consecutive runs of one mile. Each run was completed in 5 min. 30 sec. and there was a recovery period of 9 min. 30 sec. between the runs. The blood sample was taken at the end of recovery. Expired air was not collected. Plasma NEFA showed only a small rise after the first run and then remained approximately constant. It was 0.81 m-mole/l at the start and after 15 minutes at the end of recovery increased to 1.01 m-mole/l and more or less remained the same throughout. Blood glucose fell from 5.12 m-mole/l to 3.67 m-mole/l after first

TABLE VII

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 15 Subject M.J.H. Age 22 Ht. 183 cm. Wt. 104 kg.

Exercise 10 mile's walk for 145 min. (5 miles on the Meadows and 5 miles over the treadmill)

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.99	-	182	0.814
Exercise for 85 min.	2.29	-	2283	0.810
" " 115 "	2.53	-	2276	0.810
" " 145 "	2.98	-	2173	0.813
Recovery for min.				

TABLE VIII

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 16 Subject G.G.W. Age 21 Ht. 178 cm. Wt. 63.9 kg

Exercise 10 miles walk (5 miles on the Meadows and 5 miles over the treadmill)

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.96	4.28	200	0.98
Exercise for min.				
After 90 min.	1.08	3.45	-	-
" 120 "	1.67	3.00	1145	0.81
" 160 "	1.99	3.00	1590	0.85
" 180 "	2.37	3.00	-	-
Recovery for min.				

TABLE IX

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 14 Subject M.J.H. Age 22 Ht. 183 cm. Wt. 104 kg

Exercise Walk for 50 min. at 4 m.p.h. 6% gradient with 10 kg. load

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	1.04	5.47	346	0.82
Exercise for 30 min.	1.62	5.47	2677	0.91
" " 50 "	1.79	5.11	2533	0.90
Recovery for min.				

TABLE X

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 17   Subject G.G.W.   Age 21   Ht. 178 cm.   Wt. 63.9 kg.

Exercise   Three consecutive one mile runs (one mile run/5 min. 30 sec.)

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.81	5.12	289	0.86
Exercise for min.				
* After 15 mins.	1.01	3.67	-	-
After next 15 mins.	1.09	3.17	-	-
After next 15 mins.	1.09	2.84	-	-
Recovery for min.				

* After one mile run for 5 min. 30 sec., there is a recovery period of 9 min. 30 sec.

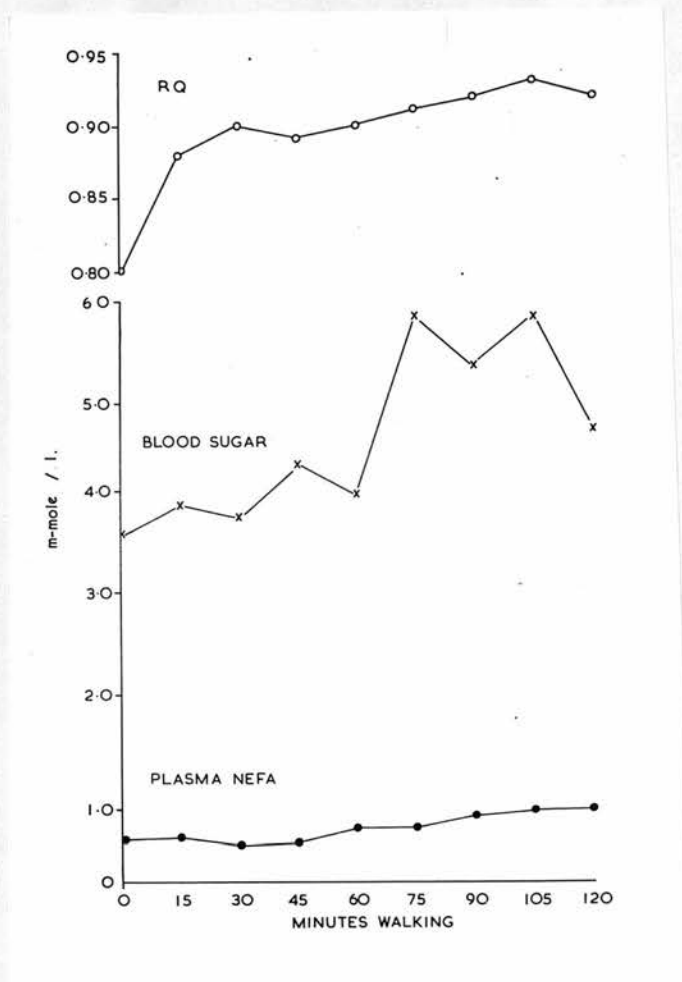

Fig. 5. The effect of glucose ingestion on walking at 4 m.p.h. in the post-absorptive state on the R.Q. blood sugar and plasma non-esterified fatty acid in a normal healthy man.

recovery and gradually decreased to 2.84 m-mole/litre.

Fig 5. shows the effect of glucose ingestion during the walk. Insignificant change was noticed in the plasma NEFA level. The change was a slight increase instead of a fall which was expected. Blood glucose increased at the end of 45 min. The plasma NEFA level remained more or less at the fasting level of 0.75 m-mole/l up to 45 minutes. Then there was a slight increase (0.86 m-mole/l) which was increased to the level of 1.01 at 105 min. Then it maintained the same level to the end of the experiment (120 min.). The blood sugar after 45 minutes increased from 3.69 m-mole/l to 4.33 m-mole/l. There was then a slow steady rise which was noted as 5.84 m-mole/l at 105 minutes and remained so up to the end of the experiment. R.Q. showed a slight rise from 0.81 to 0.93.

CHAPTER VII

DISCUSSION AND CONCLUSION

The gradual rise in the concentration of circulating NEFA with exercise found in the present investigation, can be attributed to the mobilization of fat. From the observed R.Q. it can be calculated that about 23-25 g. of fat was utilised by the subjects. It may be presumed that the presence of an increased amount of NEFA in the blood, shows that it is due to the greater utilization of fat for energy. In the present investigation there is no evidence regarding the utilization during exercise. The fall in R.Q. and lowering of blood sugar may indirectly support the use of NEFA by the muscles. However, there was no correlation between the R.Q., blood sugar level and the increased amount of plasma NEFA. It is thus rather difficult to conclude that NEFA itself was utilized by the muscles, from the present experimental data. However, there are some evidences regarding the utilization of NEFA from the experiments done by others.

Bing and his associates (1954) demonstrated that the heart extracted a great deal of fatty acids, which if completely burned could account for about 70% of the cardiac oxygen consumption. There is no doubt that

these fatty acids are NEFA. Gordon and Cherkes (1956), gave evidence that non-esterified fatty acids are the principal type of lipids extracted by the heart. Gordon et al. (1957) has shown that the human forearm usually, but not always, takes up more fatty acids than it releases. This suggests that fatty acids are directly taken up by the muscle cells. It is difficult to measure utilization of a substance directly. Measurement has been made on the basis of arteriovenous differences, which reflect the sum of a number of possible effects, such as deposition into fat depots, release from depots and uptake by muscle. However, *in vitro* work during electrical stimulation of muscle has shown utilisation of NEFA (Fritz *et al.*, 1958).

No other evidence regarding the NEFA level of plasma during exercise had been obtained, when the present investigation was done. Much later experiments during exercise were done by some other workers.

Friedberg *et al.* (1960) showed that 7 min. after the start of exercise the mean plasma NEFA in seven subjects fell from 0.85 to 0.63 m-mole/l. and remained at this level for a further 8 min. when the exercise was stopped. No measurements of plasma NEFA was done before 15 min. during the recent investigation. But at this time in four among the twelve subjects the NEFA level was below the resting level (*see* Appendix).

The data of Friedberg *et al.* (1960) indicate that exercise lowers plasma NEFA, but do not reveal whether it is due to an acceleration in the utilisation or decrease in the release from depots. They showed by means of palmitic acid 1-C^{14} that there was an accelerated removal of the NEFA. Also, they observed that there was an increase in plasma concentration of this radio-isotope immediately after exercise. They suggested there was an increase in mobilization which supports the observations of the present investigation.

Carlson and Pernow (1959), catheterised both femoral veins in their subjects and then exercised one leg only on a bicycle for short periods of time. The NEFA concentration in the plasma from the exercising leg was in all instances lower than that from the resting leg.

The above observations are all consistent with the view that the muscles can readily utilise NEFA from the plasma as a source of energy. So, it may be mobilised in excess of immediate needs and result in a rise in the plasma NEFA level.

The results of the present investigation lead to the tentative conclusion that the mobilization of fat is initiated by the factors which create a need for additional energy production. It is conceivable that the fatty acids oxidized in the cells of the peripheral tissues are those fatty acids extractable from the blood stream.

This is surely too simple a view since it is well known that lipids of tissues are undergoing turnover at significant rates.

Though the present investigation and as the work of others support the view that there is mobilization of fat in the form of NEFA during exercise, the physiological factor responsible for this is not definitely known. An attempt has therefore been made to elucidate the factor or factors responsible for the mobilisation of fat in the form of NEFA. This is described in Part II.

PART II

HORMONAL FACTORS INFLUENCING

MOBILIZATION OF NEFA

CHAPTER VIII
INTRODUCTION

SOME HORMONAL FACTORS CONCERNED WITH MOBILIZATION OF FAT

In Part I it was demonstrated that within a few minutes of the start of exercise of moderate severity mobilization of fat as NEFA occurred. It was thought that this mobilization might be controlled by the hormones as well as by the autonomic nervous system. Evidence regarding this is given below.

HORMONAL REGULATION

ANTERIOR PITUITARY EXTRACT From 1925 onwards several investigators studied the influence of anterior pituitary on fat mobilization. Best and Campbell (1936) were the first to demonstrate this influence. They showed that 3 days following an injection of an extract of the anterior pituitary gland in normal rats there occurred a reduction in depot fat followed by an increase in liver fat.

Weil and Stetten (1947) found that the urine of fasted rabbits contains a factor which causes fat accumulation in the liver, when injected into mice. No such facter was present in the urine of fed rabbits. This was named as 'adipokinin'. This could be concentrated by alcohol precipitate and was extracted from the precipitate by a method similar to those used for the extraction of anterior pituitary gland.

Campbell and Lucas (1951) stated that the reduction in carcass fat was due exclusively to a reduction in triglyceride. They could observe no reduction in cholesterol or cholesterol esters and noted a negligible change in the phospholipids. They concluded that the fate of mobilization of fat from adipose tissue, under the influence of anterior pituitary factor, was dependent on the triglyceride content of the tissue.

GROWTH HORMONE   Lee and Schaffer (1934) and Young (1945) have shown that the injection of growth-promoting anterior pituitary extracts into rats decreases the fat content of the carcass. Li, Simpson and Evans (1949) and Greenbaum (1953) demonstrated similar effects with purified growth hormone. The lowering of R.Q. and rapid mobilization of fat to the liver after administration of growth hormone, are evidence that this hormone stimulates fat metabolism. This was supported by several workers (Gaebler, 1933; Graeves, Freiberg and Johns, 1940; Greenbaum, 1953).

Weil and Ross (1949) found that doses as low as 100 µg of purified growth hormone cause a significant increase of liver fat of mice within two hours, and a doubling of the control level after 7 hours. Li, Simpson and Evans (1949) noted a similar increase when they examined the effect of acute treatment with growth hormone (5 mg. administered over a period of 6 hours) in rats

previously starved for 24 hours. In these animals the liver fat was increased by 65%. Barrett, Best and Ridout (1938) and Stetten and Salcedo (1944) have used deuterium to label the depot fats of the body. They have shown, from the rate at which deuterated fats accumulated in the liver after injections of growth hormone pituitary extracts, that the primary source of this new lipid was the body depots. Greenbaum and McLean (1953) showed that growth hormone causes a rapid mobilization of the neutral fat fraction. Raben and Hollenberg (1958) and Raben (1959) reported that growth hormone increased NEFA in the plasma. Engel et al. (1958), Knobil and Greep (1959) and Wilgram et al. (1959) observed a similar effect on the mobilization of fat.

Adrenocorticotrophic HORMONE (ACTH)

Li et al. (1949) noted that ACTH has a striking effect on the mobilization of fat in the liver.

Engel et al. (1957) showed that ACTH possesses an adipokinetic activity which was quite different from that of growth promoting and thyrotrophic hormone. They experimented on rats anaesthetised by pentobarbital sodium.

Li et al. (1957) noticed that purified α-corticotropin results, in addition to other biological effects, in the mobilization of fat into the liver of fasted mice.

White and Engel (1958) reported that the incubation of corticotropin with rat adipose tissue in rat plasma medium resulted in an increase in the concentration of NEFA in both the tissue and the incubated medium. This observation suggested that ACTH increased the rate of lipolysis of neutral fat within the cell. This was inhibited by anaerobiosis.

ADRENAL GLANDS

ADRENALINE From 1928 onwards, many investigators found that adrenaline plays an important role in fat metabolism. Cori and Cori (1928) concluded from the published evidences as well as from their own results that fat was the major fuel for the increased metabolism induced by adrenaline. Investigations in intact animals suggest that adrenaline elevated ketone production only when hepatic carbohydrate metabolism was inadequate. Clement and Schaffer (1947) and McKay (1937) injected adrenaline in normal rats and caused an increase in liver fat.

Gildea and Mann (1936) injected adrenaline to 11 normals and 5 patients, in amount sufficient to increase blood sugar and blood pressure in the subsequent two hours. They noticed a moderate, but consistent, elevation in the level of serum fatty acids. In the 9 subjects total fatty acids were increased by as much as 1 to 2.3 mEq/l.

Wool _et al._ (1954) reported that administration of adrenaline to demedullated rats restored to normal the ability to develop a fatty liver following ethionine administration.

Others showed (Kaplan and Gant, 1955) that subcutaneous injection of adrenaline in dogs, produced a hyperlipaemia within 24 hours. This suggests that adrenaline has an effect on fat mobilization. Pari passu with an action on fat transport, adrenaline increased liver lipids (Clement and Schäffer, 1947; Pollack, 1939; Wertheimer, 1926; Wool et al., 1954) and reduced adipose tissue lipid (Clement and Schäffer).

Dole (1956), Gordon and Cherkes (1956) and Anfinsen (1956) supported the view of others regarding fat mobilization. They, however, called attention to NEFA by showing that it is this fraction which is important in fat mobilization.

NORADRENALINE Until 1953 no heed was paid to the function of noradrenaline on fat metabolism. Aujard (1953) showed noradrenaline increased neutral fat but had no effect on the cholesterol or phospholipid content of blood.

White and Engel (1958a) recorded that noradrenaline stimulated production of NEFA from adipose tissue incubated _in vitro_.

THYROID GLAND. Excessive metabolism of depot fat is an obvious feature of most patients with hyperthyroidism.

Rich *et al.* (1959) showed that injection of 1-triiodothyronine results in the elevation of plasma NEFA ineuthyroid subjects. The thyroid gland also is thus concerned in fat mobilization.

INSULIN There is much evidence which suggests that insulin affects lipogenesis. Dole (1956) reported that after injection of insulin a fall in plasma NEFA occurred.

NERVOUS REGULATION The action of nervous system in the control of fat deposition and mobilization has been observed by several workers. Beznák and Hasch (1937a); Hausberger and Gujot (1937) and Clement (1947) reported that denervation of adipose tissue results in decreased fat deposition and mobilization. According to Kuré, Toshio and Okinaka (1937) the sympathetic nerves inhibit fat deposition while the parasympathetic nerves promote it.

If the nerve supply of the adipose tissue is severed, no fat infiltration of the liver could be shown. After complete denervation both fat depletion and mobilization were reduced. The denervated fatty tissue was found to possess a reduced metabolic activity (Shapiro and Wertheimer, 1948).

SOME ADDITIONAL FAT MOBILIZING SUBSTANCES

Stewart and Young (1959) have provided evidence for the existence of a ketonuria-stimulating substance from horse muscle. Chalmers, Kekwick and Pawan (1960) demonstrated the presence of a fat mobilizing substance in the urine of fasting human beings.

THE PRESENT WORK ON THE MOBILIZATION OF FAT

It is known that growth hormone and adrenaline can be responsible for fat mobilization under certain circumstances. It was therefore decided to observe the effect of moderate exercise in patients with hypopituitarism and after bilateral adrenalectomy.

An opportunity arose to work with human growth hormone and its effect in one hypopituitary patient was studied. The effect of insulin on plasma NEFA levels in diabetic patients with and without ketosis was investigated.

It is rather difficult to deduce the effect of a single hormone on cells and enzymes from experiments conducted *in vivo* because a secondary action of the hormone administered is always possible. It was thus decided to study *in vitro* the effects on adipose tissue of various hormones concerned in the fat mobilization as NEFA. Since growth hormone was found to have no effect *in vitro*, further experiments were carried out on intact rats.

CHAPTER IX

OBSERVATIONS ON HUMAN SUBJECTS

EFFECT OF EXERCISE ON NEFA LEVELS IN PATIENTS WITH HYPOPITUITARISM

It is well known that growth hormone helps in the mobilization of fat. It was therefore decided to observe the effect of moderate exercise on the mobilization in the form of NEFA in patients with hypopituitarism.

PATIENTS Two patients served as subjects. One of them, Mr. J.K. aged 53 years had been operated on for cystic epidermoid suprasellar tumour in 1931, at the age of 25. His pre-operative X-ray report stated there was a characteristically enlarged pituitary fossa and some flakes of calcification of the cyst walls above this. At this time he had presented with visual symptoms. After this he remained quite well, until 1954, when he was admitted to hospital in a drowsy state. A diagnosis was made of acute lymphocytic choriomeningitis with hypopituitary coma. In 1955 he was readmitted with a similar but more severe episode of coma. On that occasion there was no evidence of meningeal infection. In 1956 he was admitted in a drowsy state and was regarded as being in hypopituitary precoma. He responded very well to vigorous treatment with

TABLE XIV

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 23 Subject **Miss I.** Age 38 yrs. Ht. 160 cm. Wt. 46.8

Exercise **One hour walk on the treadmill at 2.5 m.p.h. with 2½% gradient**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	1.12	5.73	177	0.78
Exercise for 15 min.	1.21	5.56	717	0.83
" " 30 "	1.50	5.37	725	0.83
" " 45 "	2.05	5.28	711	0.84
" " 60 "	1.92	5.39	740	0.82
Recovery for min.				

TABLE XIII

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 22 Subject Miss I. Age 38 yrs. Ht. 160 cm. Wt. 46.8

Exercise **One hour walk on the treadmill at 2.5 m.p.h. with 2½% gradient**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	1.25	5.11	181	0.76
Exercise for 15 min.	1.65	5.11	716	0.84
" " 30 "	1.71	4.61	743	0.82
" " 45 "	2.05	4.61	748	0.82
" " 60 "	1.91	4.34	707	0.83
Recovery for min.				

TABLE XII

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 21 Subject Mr. J.K. Age 53 yrs. Ht. 157.6 cm. Wt. 78 kg

Exercise One hour walk on the treadmill at 2.5 m.p.h. with 3% gradient

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	1.22	3.78	253	0.97
Exercise for 15 min.	1.09	3.67	851	0.80
" " 30 "	1.57	3.67	1014	0.81
" " 45 "	1.66	3.07	1021	0.81
" " 60 "	1.72	3.07	1007	0.81
Recovery for min.				

TABLE XI

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 20 Subject Mr. J.K. Age 53 yrs. Ht. 157.6 cm. Wt. 78 kg

Exercise One hour walk on the treadmill at 2.5 m.p.h. with 3% gradient

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	1.18	2.72	217	1.06
Exercise for 15 min.	1.07	2.61	788	0.83
" " 30 "	1.55	2.45	941	0.83
" " 45 "	1.50	2.45	999	0.82
" " 60 "	1.57	2.72	946	0.82
Recovery for min.				

cortisone, glucose and penicillin. In recent years he has been in reasonable health and earning his living with 12.5 mg. cortisone daily by mouth. He has a fine soft skin with deficient hair growth and needs to shave only once in two months. Radiographs of the skull show much enlargement of sella turcica.

The other patient was Miss I. She was aged 38, has had evidence of pituitary insufficiency since the age of 17, when her menstrual periods ceased. She is now maintained on oral administration of cortisone 25 mg. and thyroxine 0.2 mg. daily and is able to earn her living as a seamstress and enjoys a new sense of well-being.

PROCEDURE The same procedure was followed as in the experiments with normals, save that Mr. J.K. and Miss I. walked on the treadmill at the rate of 2.5 m.p.h. with an incline of 3% and 2.5% respectively for an hour. Blood was drawn at intervals of 15 minutes and the expired air was collected at every 10 minutes during the walk, as in the experiments with healthy subjects described in Part I.

RESULTS Tables XI, XII, XIII, and XIV show the results of four experiments. The mean plasma level of Mr. J.K. was increased by exercise from 1.20 to 1.65 m-mole/l. Just after the first 15 min. of the walk a fall from 1.20 to 1.08 m-mole/l was noticed. At the end of the experiment it rose to 1.56 m-mole/l. R.Q. remained

constant at 0.82 throughout the experiment.

In Miss I., the plasma NEFA level increased from 1.19 to 1.92 m-mole/l. No fall was recorded after 15 min. as happened in Mr. J.K. Blood glucose fell from 5.42 to 4.9 m-mole/l. The R.Q. increased from 0.77 to 0.83 but it remained constant thereafter.

The results of these two experiments suggested that in spite of the absence of growth hormone the plasma NEFA level was increased. One may conclude that growth hormone is not the only factor responsible for the mobilization of NEFA.

EFFECT OF EXERCISE ON NEFA LEVELS IN ADRENALECTOMIZED PATIENTS It is known that adrenaline causes mobilization of NEFA *in vitro*. It was thought that the absence of adrenal glands might affect the mobilization. So, the following experiments were carried out.

PATIENTS Mrs. E., Mrs. D. and Miss R. had undergone bilateral adrenalectomy for recurrent cancer of the breast 7, 26 and 54 months respectively before these observations. They were aged 43, 44 and 56 years. Each was maintained on cortisone 37.5 to 50 mg. daily by mouth. They were in reasonable health, two of them looking after their young families and one being employed in an office at the time of study.

PROCEDURE This was the same as described above in hypopituitary patients, except that they walked at the

TABLE XVIII

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 27 Subject Miss R. Age 56 yrs. Ht. 156 cm. Wt. 61.6 k

Exercise Walk for an hour on the treadmill at 2¾ m.p.h. and 3.5% gradient

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.79	4.06	-	0.86
Exercise for 15 min.	0.84	3.45	858	0.84
" " 30 "	1.21	3.89	936	0.88
" " 45 "	1.52	4.06	941	0.87
" " 60 "	1.25	4.06	954	0.86
Recovery for min.				

TABLE XVII

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 25 Subject **Mr.s D.** Age **43 yrs.** Ht. **158.7 cm.** Wt. **67.0**

Exercise **Walk for an hour on the treadmill at $2\frac{3}{4}$ m.p.h. with a 3.5% gradient**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.82	3.56	220	0.70
Exercise for 15 min.	1.29	3.89	1327	0.84
" " 30 "	1.64	3.45	1310	0.80
" " 45 "	1.66	3.22	1297	0.80
" " 60 "	1.64	3.89	1254	0.80
Recovery for min.				

TABLE XVI

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 26 Subject **Mrs. E.** Age **44 yrs.** Ht. 160 cm. Wt. 63.6 k

Exercise **Walk on the treadmill for an hour at 2¾ m.p.h. with a 3.5% gradient**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.93	3.78	-	0.98
Exercise for 15 min.	1.05	4.39	1104	0.91
" " 30 "	1.28	4.00	1139	0.90
" " 45 "	-	4.34	1086	0.90
" " 60 "	1.39	4.17	1090	0.87
Recovery for min.				

TABLE XV

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 24 Subject Mrs. E. Age 44 yrs. Ht. 160 cm. Wt. 63.6 k

Exercise An hour walk on the treadmill at $2\frac{3}{4}$ m.p.h. with $3\frac{1}{2}$% gradient

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.74	5.2	202	0.72
Exercise for 15 min.	0.73	4.6	1046	0.89
" " 30 "	0.87	5.3	1138	0.91
" " 45 "	-	6.2	1078	0.90
" " 60 "	0.83	5.7	1036	0.91
Recovery for min.				

rate of $2\frac{3}{4}$ m.p.h. with an incline of 3.5%.

RESULTS Two experiments were done with Mrs. E. There was no change in the plasma NEFA level in the first experiment whereas in the second it rose from 0.93 to 1.39 m-mole/l. The blood glucose was estimated to be 5.2 to 5.7 m-mole/l and 3.78 to 4.17 m-mole/l respectively. The R.Q. was 0.72 to 0.91 in the first experiment and 0.98 to 0.87 in the second. The detailed results of these two experiments are given in Tables XV and XVI.

Tables XVII and XVIII show the results of the two studies on Mrs. D. and Miss R. respectively. In both, the plasma NEFA Level was raised. In Mrs. D. the increase was from 0.82 to 1.64 m-mole/l and in Miss R. was 0.79 to 1.25 m-mole/l. The blood sugar levels were 3.56 to 3.89 m-mole/l and 4.06 m-mole/l. respectively. In the latter almost no change was noticed in the blood sugar levels.

The results reported show that there may be some hormone other than adrenaline which is responsible for the mobilization of NEFA during moderate exercise. (Results published in Quart. J. exp. Physiol. 45: 1960, 312).

EFFECT OF HUMAN GROWTH HORMONE ON THE PLASMA LEVEL IN MAN

Evidence has been adduced which suggests that growth hormone administration produces an elevation of blood lipid concentration, a mobilization of lipid to the liver,

increased ketone body production *in vivo* and *in vitro* as well as depression of the R.Q.

Many workers (references are given in Chapter VIII) have shown that administration of physiological doses of growth hormone markedly increases the concentration of plasma NEFA. An opportunity arose to study the effect of human growth hormone in a patient with hypopituitarism.

PATIENT Miss I., the patient studied during moderate e exercise, served as the subject for the following experiment. She was admitted to hospital and ate a synthetic diet for 40 days. During this period, human growth hormone was administered. This resulted in the mobilization of fat, as evidenced by (1) a fall in the resting value of the R.Q. (2) a rise in the plasma NEFA level in the fasting state (3) increased excretion of ketone bodies. The results of the experiment have been published (J. Physiol. 1959, *148*, P39-40).

EFFECT OF INSULIN ON NEFA LEVELS IN THE DIABETICS

Insulin injection in the diabetic patients with ketosis (Bierman *et al.*, 1957, 1958) as well as in normal subjects (Dole, 1956), causes a rapid fall in the plasma NEFA leval. No evidence was gathered by these workers regarding its effect on non-ketotic patients with high blood sugar levels. As an opportunity arose, experiments were carried out with two patients, who were

non-ketotic diabetics.

PATIENTS Miss H., aged 66, height 157 cm., had noticed polyuria and thirst 12 months previously. Three months later diabetes was diagnosed, at which time she weighed 58 kg. Before admission to the metabolic ward of the Royal Infirmary a diet providing 1800 calories daily was prescribed, but she was not given insulin. She lost 5.5 kg. in weight, was losing more than 100 g. of glucose daily in the urine and blood concentrations before her mid-day meal exceeded 15 m-mole/l.

Mrs. McC, aged 54, height 150 cm., had noticed pruritus for one year before being found to have diabetes two months before the study. At that time she weighed only 46 kg. Before admission, a diet of 2000 Cal. was given, but no insulin. She lost 2 kg., excreted 80 to 100 g. of glucose daily and her mid-day blood glucose concentrations ranged from 10 to 12.5 m-mole/l.

Neither patient had ketonuria and no diabetic complications were present. The evidence of absence of ketonuria was obtained after their admission to hospital. The urine was tested with Acetest method and occasionally a positive result was recorded.

The patients were in hospital for 20 days. No observations were obtained during the first 8 days, whilst the subjects became accustomed to their surroundings and familiar with the experimental routine.

PROCEDURE For the first 5 days they were fed on a natural mixed diet and thereafter an artificial diet was substituted. This diet was a mixture of dried milk derivatives ("Complan", Glaxo), a vegetable oil emulsion ("Prosparol", Duncan Flockhart) and glucose. The gross caloric value of this diet was about 1940 per day. The ingredients for each patient were weighed out daily, blended with a measured amount of water and divided into four equal portions which were flavoured with coffee, raspberry, lemon, vanilla or peppermint, according to individual preference. They were served as milk shakes at 8 a.m., 12 noon, 4 p.m. and 8 p.m. With each of these meals 1.0 g. of sodium chloride was taken in capsule form.

The patients' day was spent in four types of activity. They were in bed just over 8 hours each night. Every morning they went for a sixty minute walk. Most of the rest of their day was spent in sitting activities, reading, knitting, watching television and in conversation. During the remainder of their time they were engaged in personal necessities such as washing, dressing etc.

METHODS

CHEMICAL ANALYSES On the 7th and 8th pre-insulin days and the 14th and 15th insulin days 20 ml. of venous blood were drawn from the ante-cubital vein at 8 a.m., 10 a.m., 12 noon, 2 p.m., 4 p.m., 6 p.m. before they

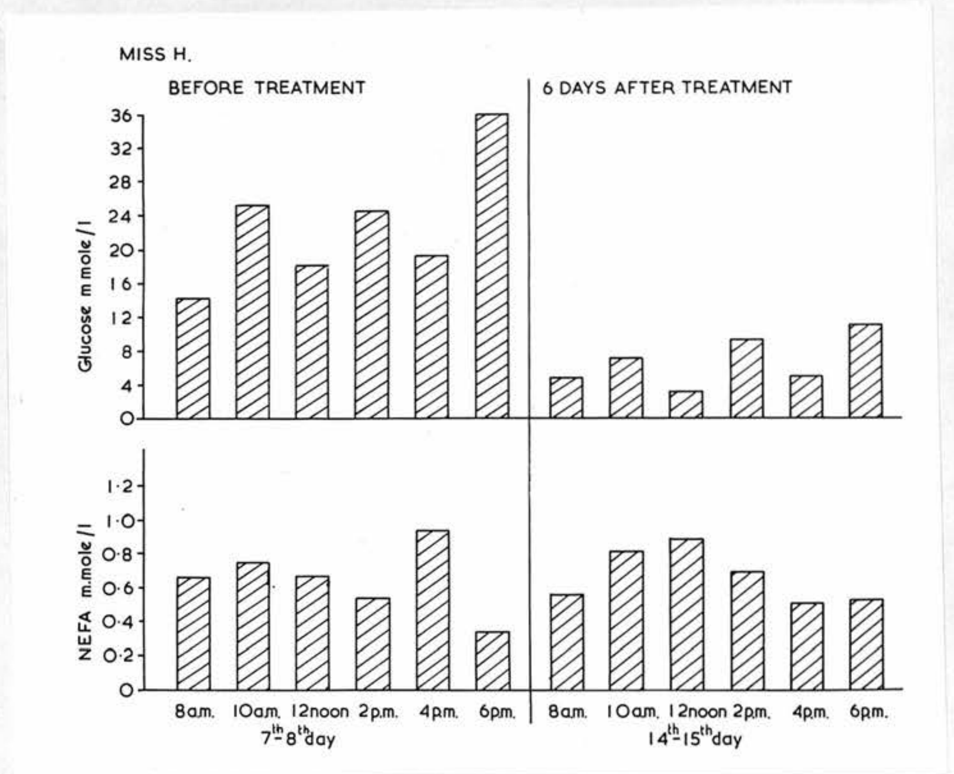

Fig. 6. Blood glucose and plasma non-esterified fatty acid level in Miss H. (diabetic patient), on a synthetic diet, on 7th-8th day before and 14th -15th day (6 days after insulin treatment.)

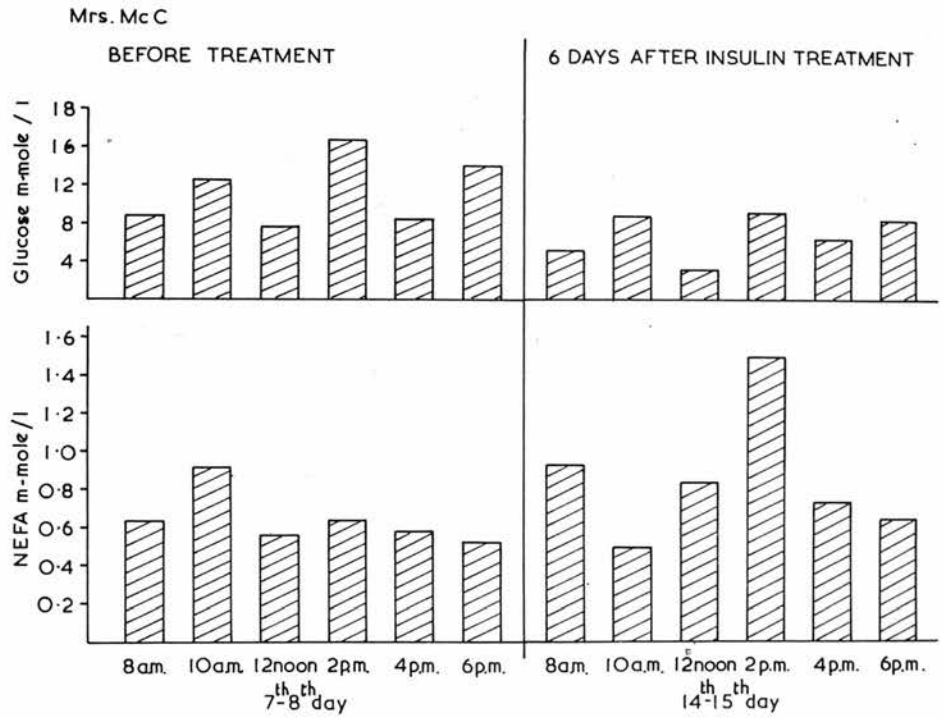

Fig. 7. Blood glucose and plasma non-esterified fatty acid level in Mrs. McC (diabetic patient) on a synthetic diet, on 7th-8th day before and 14th-15th day (6 days after insulin treatment.)

took their meals. Estimation of plasma NEFA was as described in Part I. Blood glucose was determined by a method using glucose oxidase (Hugett and Nixon, 1957). Glucose estimations were done in the hospital clinical laboratory.

<u>RESULTS</u> Figs. 6 and 7 show the blood glucose and plasma NEFA in the diabetic patients. The mean fasting blood glucose concentrations were 14.7 m-mole/l (Miss H.) and 8.5 m-mole/l (Mrs. McC). These values fell to 7.5 and 5.4 respectively during insulin therapy. Plasma NEFA was uninfluenced by insulin as shown in the Figures. The mean fasting NEFA levels in Miss H. and Mrs. McC before the treatment were 0.68 m-mole/l and 0.87 m-mole/l respectively. After insulin this became 0.75 and 0.84 m-mole/l .

<u>ADDITIONAL OBSERVATIONS IN TWO OTHER DIABETICS</u> In addition to the above mentioned observations, the plasma NEFA levels were recorded in two severely ketotic patients. These patients responded to insulin in a similar way to those reported by other workers, i.e. the blood glucose and the plasma NEFA level both fell.

<u>PATIENTS</u> Mrs. R., aged 39 years, complained of pruritus 6 months before she was admitted to the hospital (Royal Infirmary, Ward 24). Her own doctor, whosaw her at that time, prescribed a low carbohydrate diet. The pruritus was improved, but she began to lose weight rapidly. She lost 12 kg. during the next

TABLE XX

Mrs. F.

	Date	Time	NEFA m-mole/l	Blood glucose m-mole/l	Acetone (Acetest tablet)
Before Treatment	11.8.59	8 a.m.	1.47	32.7	+ +
After Insulin Treatment		12 noon	1.67	29.4	+ +
		4 p.m.	1.77	25.4	
	12.8.59	8 a.m.	1.21	6.7	+
		12 noon	1.02	10.5	-
		4 p.m.	1.53	17.3	
	13.8.59	12 noon	0.74	13.0	
	14.8.59	8 a.m.	0.88		
	15.8.59	8 a.m.	0.88		
	17.8.59	8 a.m.	0.86		
		12 noon	0.74		
		4 p.m.	0.92		

TABLE XIX

Mrs. R.

	Date	Time	NEFA m-mole/l	Blood glucose m-mole/l	Acetone (Acetest tablet)
Before Treatment					
	6.8.59	8 a.m.	1.62	18.2	+ +
		12 noon	1.60	21.4	+ +
		4 p.m.	1.95		
	7.8.59	8 a.m.	1.65		
After Insulin	10.8.59	8 a.m.	0.96	10.2	+ +
		12 noon	0.83	4.6	+ +
		4 p.m.	0.68	4.6	
	11.8.59	8 a.m.	0.92		-
		12 noon	1.17		-
		4 p.m.	1.30		
	12.8.59	8 a.m.	0.86	9.5	-
		12 noon	0.84	2.7	-
		4 p.m.	0.64	4.6	
	13.8.59	12 noon	0.50		-
	14.8.59	8 a.m.	1.01		-
	15.8.59	8 a.m.	0.86		-
	17.8.59	8 a.m.	0.86		-
		12 noon	0.91		-
		4 p.m.	0.91		-
	20.8.59	12 noon	0.76		
		4 p.m.	0.83		
	22.8.59	8 a.m.	0.69		-

6 months and felt progressively tired. For 6 weeks, she has had severe thirst, polyuria and her vision became blurred. Her fasting glood glucose level was 18.2 m-mole/l and the mean fasting plasma NEFA level was 1.62 m-mole/l on the admission to hospital. The fasting blood glucose level fell to 9.8 m-mole/l and the mean plasma fasting NEFA level to 0.88 m-mole/l as a result of insulin treatment.

Table XIX shows the blood levels of glucose and NEFA and excretion of ketone bodies by the Acetest method at 8 a.m., 12 noon and 4 p.m. As glucose estimation was at the hospital clinical laboratory, only a few estimations were done in comparison to those of plasma NEFA.

Mrs. F., aged 69 years, complained of polyuria and polydypsia for 6 months. Her fasting blood glucose was 32.7 m-mole/l on admission and after insulin treatment it fell to 6.7 m-mole/l. The fasting plasma NEFA level fell from 1.47 to 0.70 m-mole/l. Table XX shows the blood glucose and plasma NEFA levels and excretion of ketone bodies.

CONCLUSION The observatinns on two non-ketotic and two ketotic diabetics suggested that the plasma NEFA level was influenced by insulin therapy in the latter but not in the former.

CHAPTER X

EXPERIMENTS IN VITRO

The most significant source of NEFA in any quantitative sense is adipose tissue. It is as important in relation to fat, as the liver to carbohydrate metabolism. When incubated at 37°C it releases NEFA into the surrounding medium, whether this contains serum or serum substitute (e.g. Krebs medium) provided that serum albumin is present.

METHODS AND MATERIALS Epididymal fat pad from albino rats of Wistar strain was used. The rats weighed from 150-250 g. The day prior to the experiment they were starved for 20-24 hours, only water being given. Tuerkischer and Wertheimer (1946), showed that after 16-18 hours no glycogen could be detected in the adipose tissue. At the time of sacrifice in the present investigations there could thus be hardly any glycogen in the epididymal fat pad.

The rats were sacrificed by gas. Within five minutes after their death, the lower abdominal cavity was exposed after gently displacing the skin. Then, thin ends of each epididymal fat pad were seized lightly with smooth forceps, elevated until the main epididymal vessels could be seen. The adipose tissue was removed

TABLE XXI

KREBS-RINGER-PHOSPHATE BUFFER

NaCl (0.9)%	100 ml.
KCl (1.15)%	4 ml.
$MgSo_4 \cdot 7H_2O$ (3.82)%	1 ml.
*Na_2HPO_4 (0.25M)) HCl (1N))-	21ml.

*Na_2HPO_4 and HCl are made up to 100 ml. with distilled water by mixing 40 ml. and 2 ml. respectively.

Amount of cations and Anions present

	mEq/1
Na^+	133.68
K^+	4.44
Mg^{++}	2.22
	140.34
Cl^-	126.62
$HPO_4^=$	11.50
So_4	2.22
	140.34

by a single cut across the base just above the level of these vessels. The tissue was then removed to a Petri dish containing 0.9% sodium chloride solution, in order to be rinsed free from blood. This solution was at room temperature (about 20°C).

Usually, before incubation of tissues, ice-cold normal saline is used. It was found in the present investigation that excessive handling and chilling result in the diminished liberation of NEFA during incubation. Each pad was cut into small pieces and each piece was weighed separately on a previously weighed watch glass. Before it was placed on the watch glass, its water was blotted off by means of a filter paper. The object of weighing the pieces on watch glass is to keep their weights within a normal range of 90-100 mg. Then, the piece was transferred to a previously weighed Warburg's bottle containing medium in the flask and serum albumin solution in the side tube. The piece was re-weighed along with the Warburg's bottle. This final weighing gave the actual weight of the piece. Pieces from the same side were used, if possible. The whole operation took about 25 min. The medium was the Krebs-Ringer -phosphate buffer, the composition of which is given in Table XXI. (Krebs, 1933, Calcium omitted).

The pH of this solution was adjusted to 7.4 by adding 0.1 N NaoH and checked with a Pye pH meter. The flask contained 2 ml. of this solution while the side tube contained 1 ml. of 5% serum albumin solution in medium.

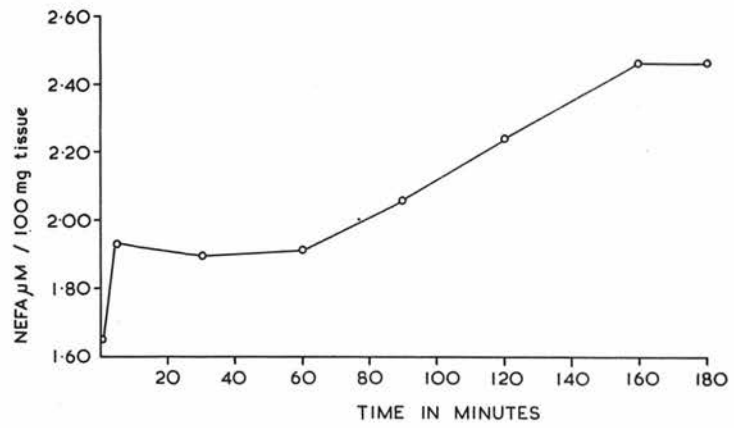

Fig. 8. <u>See</u> text

The bottle thus contained 3 ml. of liquid. The serum albumin was kept in the side tube until the bottles were placed in the bath for incubation. It was observed that as soon as the tissue came in contact with serum albumin it started to release NEFA. Just before the incubation was begun, the albumin solution was tipped into the body of the flask.

Albumin was found to be obligatory for the release of NEFA. It was also required for lipolysis. In its absence both release and lipolysis stop. The serum albumin used in the present experiments was the bovine fraction V of Armour & Co. This material contained some NEFA in combination which was estimated by setting up a 'blank' of serum albumin before estimation of NEFA released by the incubated adipose tissue.

Reshef et al. (1958) showed that the capacity of the mesenteric tissue to release NEFA decreases with time and ceases after approximately 150 min. incubation. There was no such report recorded in the case of the epididymal fat pad. Most workers, however, have done experiments for 3 hours. Likewise, in the present investigation, the adipose tissue was incubated for 3 hours. Fig. 8. shows that the maximum release of NEFA had occurred within 150 min. and did not increase further up to 180 min. The tissues were incubated in

under air. As the tissue might contain some preformed NEFA, it was decided to determine the NEFA content of tissue which was not incubated. This preformed NEFA was mentioned as 'Initial NEFA'. It was estimated as follows. Tissues which were not incubated, after being weighed on the watch glass were replaced in beakers containing 3 ml. Krebs-Ringer-phosphate-buffer. The beakers and solution were weighed before and after addition of the tissue. Then the fluid was filtered through glass wool into centrifuge tubes. The tissues were then transferred to dry and clean centrifuge tubes. To each 3 ml. of 5% serum albumin in Krebs-Ringer-phosphate buffer were added and stirred with a glass pipette for 30 sec. After that, they were left standing on the bench at the room temperature (about 20°C) for 15-20 min. The material was again filtered as before. Some of the tissues after being treated with serum albumin were homogenized with water and the materials were analysed in order to find out if there was any NEFA inside the tissue after extraction with serum albumin. It was found that the tissue contained a negligible amount of NEFA. The process of measurement of tissue NEFA was therefore discontinued after the extraction with serum albumin. The filtered samples were left well corked on the bench and the NEFA analysed along with the

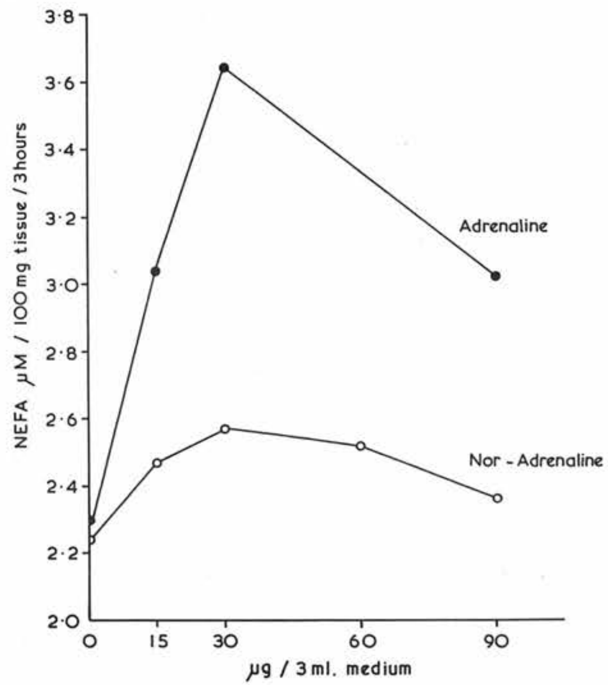

Fig. 9. See text

incubated materials. All samples were analysed in triplicate.

EFFECT OF ADRENALINE AND NORADRENALINE

METHODS AND MATERIALS Epididymal fat was treated with l-adrenaline and l-noradrenaline. Adrenaline was the adrenaline chloride solution (1:1000 'Adrenalin') of Parke Davis. Each ml. contained 1 mg. adrenaline as the hydrochloride dissolved in physiological salt solution. Noradrenaline used was known as 'Levophed' of Bayer production. Each ml. contains 1 mg. l-noradrenaline as the bitartrate.

DOSAGE 15, 30, 60 and 90 µg. were used per 3 ml. medium. Amounts less than 15 µg/3 ml. produced no effect. The minimum dose used was therefore 15 µg. From 15 µg. to 60 µg. NEFA liberation increased. Above 60 µg. a fall was recorded in the NEFA concentrations; no doses above 90 µg. were therefore used.

RESULTS Fig. 9. shows the effects of both the hormones on the release of NEFA in 5 to 11 observations. Both induce a steady rise of NEFA from 15 µg. to 30 µg./3 ml.; a fall was recorded after 60 µg./3 ml. in each of the hormones. In adrenaline treated tissues the rise was more pronounced than the noradrenaline treated tissues.

However this power of lipolysis by adrenaline as well as noradrenaline is similar to that of White and Engel (1958a) and Gordon and Cherkes (1958). The only

TABLE XXIV

Effect of ACTH on the non-esterified fatty acids released by epididymal fat *in vitro* (μmole/100 mg. tissue/3 hr.)

Time of incubation	ACTH (IU/3ml)	NEFA released
0	0	1.93
3 hr.	0	2.47
3 hr.	0.6	2.43
3 hr.	0.9	2.41
3 hr.	1.8	2.50
3 hr.	3	2.45

(mean values for 3 experiments)

TABLE XXIII

Effect of noradrenaline on the non-esterified fatty acids released by epididymal fat *in vitro* (μmole/100 mg. tissue/3 hr)

Time of incubation	l-noradrenaline (μg/3 ml.)	NEFA released
0	0	1.90 ± 0.17
3 hr.	0	2.24 ± 0.17
3 hr.	15	2.47 ± 0.17
3 hr.	30	2.57 ± 0.40
3 hr.	60	2.52 ± 0.21
3 hr.	90	2.36 ± 0.20

(all figures mean values ± standard deviation of 5 to 11 observations)

TABLE XXII

Effect of adrenaline on the non-esterified fatty acids released by epididymal fat *in vitro* (μmole/100 mg. tissue / 3 hr.)

Time of incubation	l-adrenaline (μg/3 ml.)	NEFA released
0	0	1.75 ± 0.28
3 hrs.	0	2.29 ± 0.40
3 hrs.	15	3.04 ± 0.61
3 hrs.	30	3.65 ± 1.13
3 hrs.	90	3.12 + 0.94

(all figures mean values ± standard deviation of 9 observations)

difference between the observations made by these two groups of workers and the present experiments was that a fall was recorded as the dosage was increased. This fall might be accounted for by the increased fatty acid oxidation by the increased amount of the hormones.

The amounts of NEFA liberated from the adipose tissue by the action of adrenaline and noradrenaline was 1.75 to 3.65 μ-mole/100 mg./3 hr. and 1.90 to 2.57 μ-mole/100 mg./3 hr. respectively. The detailed result is given in the Tables XXII and XXIII.

EFFECT OF OTHER HORMONES ON MOBILIZATION

CORTICOTROPHIN (ACTH) To note the effect of ACTH on the adipose tissue, experiments were done with Crookes corticotrophin powder. This can be obtained in vials containing 10, 25 or 50 I.U. Vials of 10 I.U. were used in the present experiments. The powder was dissolved in Krebs-Ringer-phosphate buffer.

DOSAGE The human experiment by Laurell and Christensson (1958) with 10 I.U. of ACTH suggested that the following amounts of ACTH might be effective in the liberation of NEFA from the adipose tissue. The dosages of ACTH used in the present experiment were 0.3, 0.6, 0.9, 1.8 and 3 I.U.

RESULT Table XXIV records the effects of 3 experiments done with ACTH. No change was noted.

TABLE XXV

Effect of cortisone on the unesterified fatty acid released by epididymal fat *in vitro* (μmole/100 mg. tissue/3 hr.)

Time of incubation	Cortisone (mg/3ml)	NEFA released
0	0	1.93
3 hrs.	0	2.36
3 hrs.	0.75	2.30
3 hrs.	1.5	2.32
3 hrs.	3 mg.	2.39

(mean values of 3 experiments)

CORTISONE Cortelan (Glaxo) was used. This can be obtained in vials containing 25 mg. cortisone acetate per ml. The amounts added to the medium were as follows: 0.75, 1.5 and 3 mg./3 ml. medium.

RESULT Table XXV shows the effect of Cortelan in varied doses. From three experiments, negative results were obtained.

THYROID GROUP OF HORMONES 3.5.3′ tri-iodo-l-thyronine was used. In intact rats 10 μg. of this product raised the heart rate and oxygen consumption. (Dr. Boyd, personal communication). As it cannot be dissolved in water or in the medium, it was dissolved at first in 0.002 N NaoH at room temperature (20°C approx.).

The volume was then made up to the required amount by adding the medium. It was known that these hormones never worked *in vitro*, as they work inside the body after their conversion into a simpler breakdown product. To confirm this, one experiment was done with dosages varying from 0.75 μg. to 3 μg./3 ml. No effect was recorded.

GROWTH HORMONE This affects the mobilization of fat by increasing the output from adipose tissue, as mentioned in the chapter of Introduction p. 37-38. This can be supported by some of the experimental observations of other workers. Brown and Benett (1959) measured the conversion of ^{14}C labelled palmitic acid to $^{14}CO_2$ in

human subjects with and without this hormone. The percentage of the label appearing as $^{14}Co_2$ was not changed by the hormone, but as there was a greater amount of plasma NEFA after the administration of the hormone, the total conversion of fatty acid to Co_2 was shown to be greater after the hormone. Knobil (1959) incubated adipose tissue removed from hypophysectomized rats and found an increased release of NEFA from fat from animals receiving growth hormone. This was determined by the total amount of NEFA present in the tissue and medium at the end of incubation. In the present experiments the effect of growth hormone on adipose tissue was studied _in vitro_.

METHODS AND MATERIALS Adipose tissue was used. The bovine growth hormone (NIH) was obtained from Clinical Endocrinology Research Unit, Edinburgh and Professor Wilhelmi (Atlanta). The amount used varied from 0.3 to 3 mg./3 ml. medium. As it is difficult to dissolve in distilled water (Lazo-Wazem _et al._, 1958), it was first dissolved in a drop or two of 0.1 N NaoH. Growth hormone is soluble either in acid or alkali. After it was dissolved in alkali, Krebs-Ringer-phosphate buffer was added to make it up to the required volume. Most of the workers have failed to find an action of growth hormone _in vitro_ (White and Engel, 1958b; Krahl, 1955), but there has been some experiments in

TABLE XXVI

Effect of one sample of growth hormone on the non-esterified fatty acids released by epididymal fat *in vitro* (umol/100 mg. tissue/3hr.)

Time of incubation	Growth hormone added (mg/3ml.)	NEFA released
0	0	1.92
3	0	2.76
3	1.5	3.40
3	0.75	3.24

which a positive result has been recorded. In the present investigation, 6 experiments were done with three different growth hormone preparations. In 5 of these there was no effect, but in one there was a positive effect, which is shown in the Table XXVI. The failure of growth hormone to act in vitro in the two samples, lead one to think that like insulin age may be important for the action of the former hormone. Hagen et al. (1959) showed that insulin fails to work on the tissue of adult rat. Small young rats weighing below 100g. were therefore selected. Even the experiment carried out with growth hormone in vitro gave a negative result. The experiments described in Chapter XI were therefore planned.

CHAPTER XI

EXPERIMENTS ON INTACT RATS

The failure of growth hormone to act *in vitro* suggested that intact rats should be used. It was considered that growth hormone may act through the intervention of the sympathetic nervous system and that the absence of the nerves in the excised adipose tissue may be concerned with the inactivity of growth hormone. If the action of this hormone depended upon the sympathetic nervous system, the effect might have been immediate. Some experiments were therefore carried out to note the time of action of growth hormone. An increase in plasma NEFA level four hours after the administration of the hormone was shown by previous workers.

Raben (1957) reported that after intravenous administration of 4 mg. of acetic acid extracted human growth hormone to a hypopituitary patient, no rise of NEFA was found in an hour, but there was a four-fold increase after four hours. No previous literature is available concerning the effect before 4 hours.

It was also known that doses of growth hormone from 1 mg. to 5 mg. per 100 g. are effective in a rat, yet the minimum effective dosage is not known. With these considerations in mind the minimum effective dosage was determined.

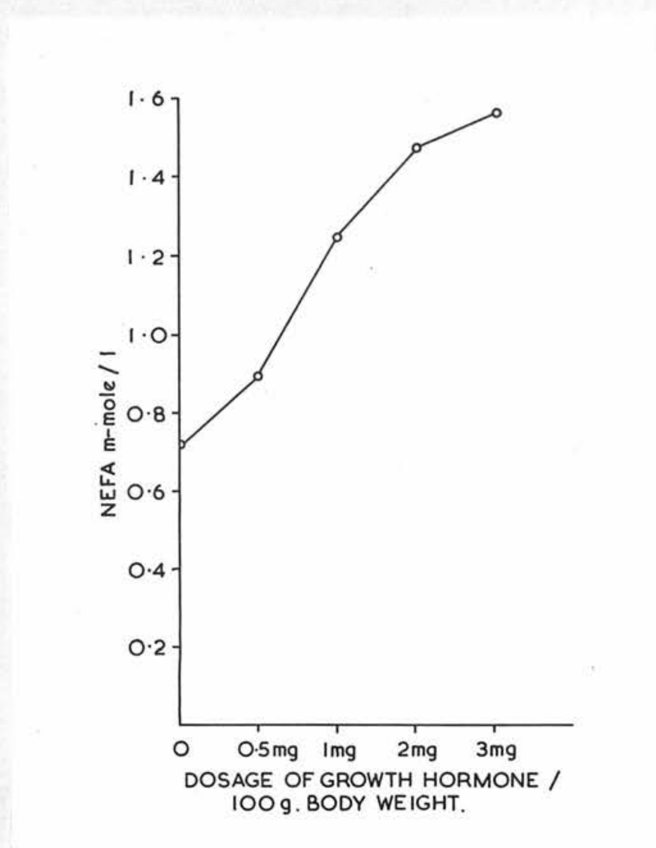

Fig. 10. Plasma non-esterified fatty acid levels in fasted rats, after injection of graded doses of growth hormone per 100g. body weight after a period of 4 hours.

To this end the present experiments were done to record the time and dosage of growth hormone, which is effective in the rats.

EXPERIMENTAL ANIMALS Male rats of Wistar strain were used. The weights of the animals varied from 150-250 g. They were fed on rat cakes and bread and milk. The day before the experiment they were starved for 20-24 hours.

GROWTH HORMONE Bovine growth hormone was used as in the *in vitro* work. It was suggested in 1955, that the discrepancy in its activity in man might be due to a species specificity of the hormone (Wilhelmi, 1955). Data collected since then support this view. Human growth hormone was shown to be metabolically active in man. The species specificity of the hormone is not confined to man but applies also to some other species. In general, it can be stated that growth hormone prepared from pituitary glands of lower animals is inactive in the higher species, whereas growth hormone from higher species is active in the lower animals. Biological testing showed that human and bovine hormones are equally active in hypophysectomized rats.

EFFECT OF DIFFERENT DOSAGES Growth hormone in doses of 0.25 mg. to 3 mg./100 g. body weight were injected intraperitoneally. Fig. 10 shows the concentration of plasma NEFA during a study lasting four hours. 0.5 mg./ 100 g. body weight had a slight effect on plasma NEFA

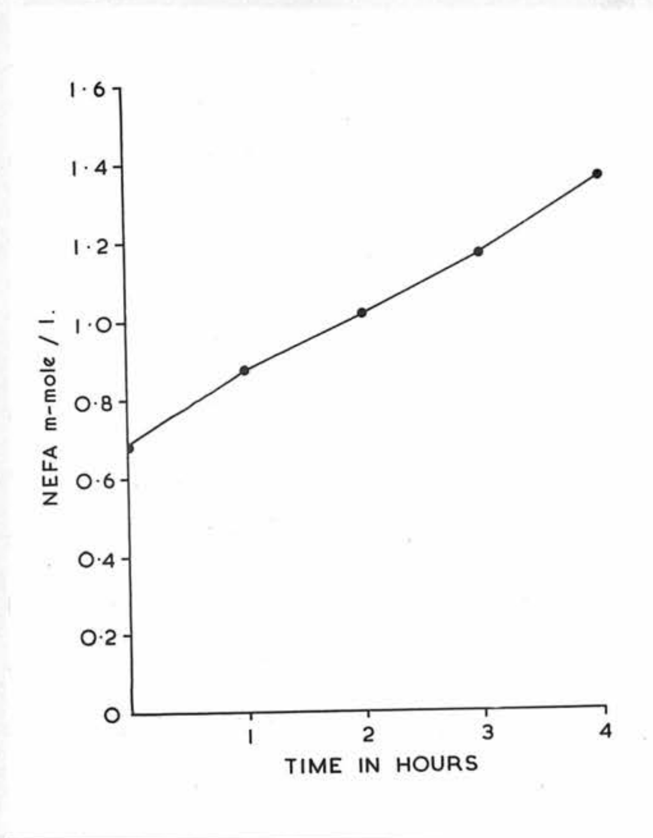

Fig. 11. Plasma non-esterified fatty acid levels in fasted rats, after injection of 1mg. growth hormone per 100g. body weight at different time periods.

level, but 1 mg./100 g. body weight had a pronounced effect. The mean NEFA level rose from 0.72 to 1.25 m-mole/l. Details are given in Table XXVII.

The experiments were done with twenty rats. As there was slight increase only after 0.5 mg. and a bigger rise after 1 mg. per 100 g. body weight, the latter was considered to be the minimum effective dose.

EFFECT OF TIME ON THE PLASMA NEFA LEVEL The minimum effective amount of growth hormone was injected intraperitoneally into 16 rats and groups of 4 rats killed by decapitation at 1 hour, 2 hours, 3 hours, and 4 hours. Four rats served as controls. Fig. 11 shows the results in 20 rats. After an hour the rise was insignificant. From two to four hours the plasma NEFA level was gradually increased being doubled at the fourth hour. The mean fasting NEFA level rose from 0.69 to 1.35 m-mole/l at 4 hr. after growth hormone injection. Details are given in Table XXVIII.

CONCLUSION The presence of a long latent period suggests the effect of growth hormone may not be mediated through the sympathetic nervous system.

TABLE XXVII

EFFECT OF INCREASED DOSAGES OF GROWTH HORMONE

ON INTACT RAT IN MOBILISING FAT AS NEFA

No. of experimental animals	Hormone injected (mg/100g. body weight)	Plasma NEFA level in rats (m-mole/l)			
		Experiment No.			
		I	II	III	IV
4	0	0.712	0.720	0.740	0.700
4	0.25	0.716	0.730	0.680	0.800
4	0.50	0.878	0.891	0.891	0.888
4	1.0	1.172	1.254	1.230	1.380
2	2.0	1.430	1.551		
2	3.0	1.548	1.588		

TABLE XXVIII

EFFECT OF TIME ON MOBILIZATION OF PLASMA NEFA

ON INTACT RATS BY GROWTH HORMONE

No. of experimental animals	Hormone injected (mg/100g. body weight) Hours after injection.	Plasma NEFA level in rats (m-mole/1) Experiment No.			
		I	II	III	IV
4	0	0.677	0.759	0.594	0.716
4	1	0.825	0.759	0.759	0.776
4	2	1.056	1.122	0.957	0.924
4	3	1.023	1.089	1.254	1.304
4	4	1.386	1.518	1.172	1.419

CHAPTER XII

DISCUSSION

The experiments of Part I lead to the conclusion that moderate exercise causes mobilization of fat as NEFA. Then the question of mobilization was partly solved in Part II.

The various factors concerned in mobilization such as growth hormone, adrenaline, noradrenaline and insulin did not altogether exclude the idea of one lipo-kinetic substance.

It is quite certain that adrenaline and noradrenaline act in the mobilization of NEFA, though moderate exercise in the bilaterally adrenalectomized patient showed that it may not be essential. There is much evidence regarding the effect of adrenaline and noradrenaline on mobilization of fat from adipose tissue *in vitro*. Also, there is evidence that the excretion of Catechol amines (von Euler and Hellner, 1955; Holmgren, 1956) as well as the plasma concentration of adrenaline and noradrenaline (Crag and Beetham, 1957) are increased during exercise. Thus the action of adrenaline was not completely ruled out. Yet the action of growth hormone in intact rats shows that it is not the only lipo-kinetic substance.

The delayed action of growth hormone after administration to rats in the present investigation suggest that this action is not mediated by a release of adrenaline. The failure of the growth hormone to work *in vitro*, led one to think that the absence of sympathetic nervous supply to the excised adipose tissue might be the cause. For, Clement (1937) and Wertheimer and Shapiro (1948) were of the opinion that the sympathetic innervation of adipose tissue is important for normal storage and transport.

That the action of growth hormone is not mediated by a release of adrenaline is also suggested by the inability of hypophysectomized monkeys to respond to exogenous adrenaline by an increase in plasma NEFA concentration (Goodman and Knobil, 1959).

The failure of growth hormone to act *in vitro* as well as the experiments with hypopituitary patients, resulting in the increase of plasma NEFA level during moderate exercise raises the possibility of some other hormone being concerned with the high plasma NEFA level. This does not disprove the action of this particular hormone, for its failure *in vitro* might be explained by conversion of growth hormone into a different compound in the body before it exerts some of its effects (Krahl, 1955). The hormone may also stimulate the production of other fat-mobilizing substances.

It may stimulate the anterior pituitary gland, for it was found by several workers (references given in the introduction chapter) that this gland is the agent responsible for fat mobilization during fasting.

According to Levin and Farber (1952) the 'fat-mobilizing hormone' is not identical with ACTH as neither ACTH nor cortisone alone was able to induce transfer of fat to the liver.

Young and Stewart (1959) mentioned a ketopoietic substance present in horse muscle.

Recently, Chalmers, Pawan and Kekwick (1960) demonstrated the presence of a polypeptide-like substance in the urine of people who are actively mobilising and utilising fat. This substance caused transient hypoglycaemia, ketonaemia and increased mobilization and catabolism of fat in mice, with depletion of body fat stores. At a concentration of less than 1 ug. per ml. the material releases non-esterified fatty acids from rat adipose tissue *in vitro*. The pituitary gland is supposed to be concerned with its production. It is apparently neither growth hormone nor ACTH.

All this evidence, as well as the present investigation, leads one to conclude that several factors become active during phases of increased energy requirements. These factors may be triggered by one lipokinetic substance to affect the plasma NEFA according to cellular requirements.

ACKNOWLEDGEMENTS

I owe a particular debt to Professor D. Whitteridge F.R.S. and to Dr. R. Passmore, F.R.S.E. Apart from affording me facilities and advice most generously, they provided the homely atmosphere in which a student from abroad could work and gain some insight into the methods of physiological research.

For the help given me by the technical staff, especially by Mr. D. Shirling of the Department, I am most grateful.

Dr. E.A. Harris, of the Department of Medicine and Therapeutics, Royal Infirmary, Edinburgh, gave valuable advice and help, to whom I am also indebted.

APPENDIX I

Tables of Individual Experiments of Exercise

EXPERIMENT 2

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 2 Subject A.B. Age 32 Ht. 160 cm. Wt. 73.2 kg.

Exercise **Walk for an hour at 3 miles per hour on treadmill.**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	1.26	4.36	202	0.80
Exercise for 30 min.	1.76	3.17	966	0.84
" " 60 "	1.88	3.28	909	0.84
Recovery for min.				

EXPERIMENT 5

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 5 Subject R.J.C. Age 22 Ht. 176 cm. Wt. 70 kg.

Exercise for one hour walking at 4 m.p.h. on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.61	4.81	243	0.73
Exercise for 15 min.	0.82	5.00	1195	0.78
" " 30 "	0.76	4.78	1137	0.76
" " 45 "	0.86	4.17	1137	0.75
" " 60 "	1.10	3.89	1147	0.76
Recovery for min.				

EXPERIMENT 6

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 6 Subject M.H.D. Age 38 yrs. Ht. 178.8 cm. Wt. 65.5kg.

Exercise Walk for an hour at 4 m.p.h. on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.69	-	196	0.83
Exercise for 15 min.	0.63	-	1380	0.81
" " 30 "	0.84	-	1264	0.79
" " 45 "	0.91	-	1352	0.78
" " 60 "	0.98	-	1284	0.78
Recovery for min.				

EXPERIMENT 7

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 7 Subject K.B.R. Age 36 yrs. Ht. 181.3 cm. Wt. 79.5 kg.

Exercise **Walk for an hour at 4 m.p.h. on treadmill**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.61	6.06	262	0.90
Exercise for 15 min.	0.97	5.62	1627	0.86
" " 30 "	0.68	4.00	1506	0.86
" " 45 "	0.88	5.00	1482	0.85
" " 60 "	1.02	4.00	1488	0.87
Recovery for min.				

EXPERIMENT 8

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 8 Subject R.P. Age 49 Ht. 182 cm. Wt. 63 kg. 35 g.

Exercise 4 miles /hr. for 60 minutes on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.80	4.2	247	0.84
Exercise for 15 min.	0.76	4.1	1195	0.87
" " 30 "	1.16	3.34	1215	0.85
" " 45 "	1.45	3.11	-	-
" " 60 "	1.53	3,11	1256	0.82
Recovery for min.				

EXPERIMENT 9

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 9 Subject S.L. Age 27 yrs. Ht. 168 cm. Wt. 60 kg.

Exercise **Walk for an hour at 3 m.p.h. on treadmill**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.64	4.5	204	0.78
Exercise for 15 min.	1.01	4.4	1132	0.87
" " 30 "	1.14	4.0	1096	0.86
" " 45 "	1.41	4.4	1150	0.89
" " 60 "	1.38	4.4	1127	0.88
Recovery for min.				

EXPERIMENT 10

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 10 Subject P.G. Age 21 yrs. Ht. 168 cm. Wt. 65.1 kg.

Exercise Walk for an hour at 3 m.p.h. on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.67	4.58	-	0.78
Exercise for 15 min.	1.02	3.89	1193	0.81
" " 30 "	1.19	3.89	1128	0.80
" " 45 "	1.42	4.31	1240	0.75
" " 60 "	1.60	3.89	1260	0.76
Recovery for min.				

EXPERIMENT 11

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 11 Subject L.T. Age 23 Ht. 177 cm. Wt. 63.7 kg.

Exercise Walk for an hour at 3 m.p.h. on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.69	5.49	1965	0.79
Exercise for 15 min.	1.06	5.34	1181	0.82
" " 30 "	1.34	5.34	1134	0.78
" " 45 "	1.71	5.01	1081	0.77
" " 60 "	2.01	5.93	1072	0.76
Recovery for min.				

EXPERIMENT 12

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 12 Subject M.B. Age 21 Ht. 182 cm. Wt. 61.0 kg.

Exercise An hour walk at 4 m.p.h. on treadmill

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.84	3.53	290	0.68
Exercise for 15 min.	0.91	3.49	1287	0.80
" " 30 "	1.05	3.40	1137	0.75
" " 45 "	1.98	2.98	1166	0.75
" " 60 "	1.81	-	1224	0.76
Recovery for min.				

EXPERIMENT 19

Effect of Exercise on the Level of Non-esterified Fatty Acids in the Plasma and Glucose in the Blood and on Oxygen Consumption.

Experiment No. 19 Subject M.H. Age 24 Ht. 170 cm. Wt. 63.5 kg.

Exercise **An hour walk at 4 m.p.h. on treadmill**

	NEFA m-mole/litre	Glucose m-mole/litre	O_2 used ml/min	R.Q.
Basal	0.73	3.80	292	0.72
Exercise for 15 min.	0.83	3.54	1333	0.76
" " 30 "	-	-	1227	0.86
" " 45 "	0.94	4.15	1218	0.87
" " 60 "	1.089	4.06	1238	0.85
Recovery for min.				

APPENDIX II

\- Reprint from J. Physiology

[*From the Proceedings of the Physiological Society*, 10–11 July 1959.]
Journal of Physiology, **148**, 39–40*P*.

The effects of a human growth hormone preparation on man.

By A. Basu, R. Passmore and J. A. Strong. *Departments of Physiology and Medicine, University of Edinburgh, and the Western General Hospital*

A primary effect of growth hormone on rats is to act as a specific stimulator of fat katabolism (Greenbaum, 1953). We have been able to demonstrate this effect in man, using a human growth hormone preparation. The subject was a woman, aged 38, who had had evidence of pituitary insufficiency for many

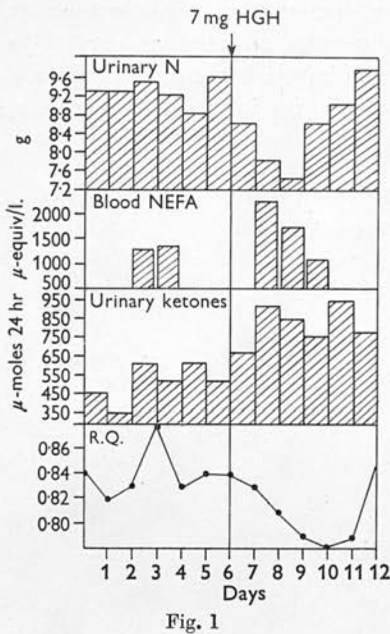

Fig. 1

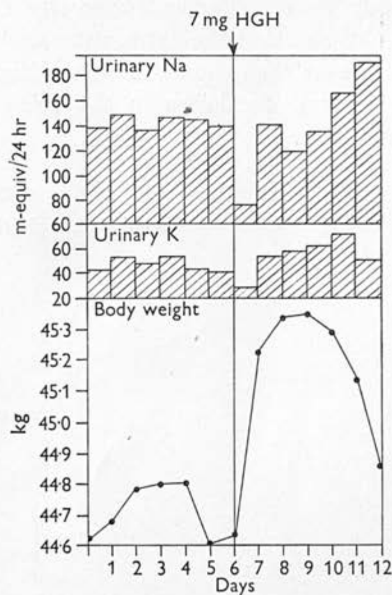

Fig. 2

Fig. 1. Changes in the basal R.Q., in the urinary excretion of ketones and nitrogen and in the level of non-esterified fatty acids in the blood following an injection of a preparation of human growth hormone.

Fig. 2. Changes in body weight and in the urinary excretion of sodium and potassium following an injection of a preparation of human growth hormone.

years. After a period of study to establish the diagnosis maintenance therapy was started with cortisone and thyroxine. Six weeks later she was admitted to hospital and kept on a constant formula diet, which exactly met the caloric requirements of a standardized regimen of physical activity. She continued to take the same doses of cortisone and thyroxine, but no other drug. After one injection of a preparation of human growth hormone, evidence of increased fat metabolism was provided by: (1) a fall in the resting value of

[P.T.O.

the R.Q., (2) a rise in the urinary excretion of ketone bodies, and (3) a rise in the level of non-esterified fatty acids in the blood in the fasting state. Associated with these changes there was a fall in urinary nitrogen, presumably due to the sparing action of the increased fat metabolism on protein metabolism. Figure 1 shows these changes and indicates that they started within 24 hr of the injection and were at a maximum 3–5 days later.

Changes in the water and electrolyte balances have followed the injection of growth hormone. There was retention of both sodium and potassium, shown by falls in urinary output, and also retention of water, shown by a sharp rise in body weight. Figure 2 indicates that these changes took place in the first 24 hr, and that the extra water and electrolytes were retained for 3 or 4 days and were then excreted. In addition, after the injection there was increased excretion of calcium in the urine and a small rise in basal oxygen consumption.

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