dissociated cells (Harris, 1955*a*). It was found that after the cells had been dissociated and had again become adherent to the glass surface on which they were to be grown, only 10 % of them or less were in the phase of DNA synthesis. During the next 36 hr. the percentage of cells synthesizing DNA rose rapidly and later fell rapidly. The fluctuations in the proportion of labelled cells showing nucleolar labelling during this period are set out in Fig. 5. The cells were exposed to tritiated thymidine for 5 min. periods at the times shown. It will be seen that as increasing numbers of cells move into the phase of DNA synthesis the percentage of labelled cells showing nucleolar labelling rises; and as increasing numbers move out of the phase of DNA synthesis the percentage showing nucleolar labelling falls. This is the case both for the stage represented by Plate 1*b* and that represented by Plate 1*c*. Conversely, the percentage of labelled cells showing no nucleolar labelling (Plate 1*d*) falls as increasing numbers of cells move into the phase of DNA synthesis and rises as increasing numbers move out of it. It follows then that the stages showing nucleolar labelling precede the stage showing no nucleolar labelling and that the probable sequence of events is represented by Plate 1*b*, *c* and *d*, in that order. There are therefore good grounds for concluding that the process of DNA synthesis is initiated at the nucleolus. Too little is known about the nucleolus to permit useful speculation at this stage about the possible biochemical role which it might play in this connexion; this problem is under current investigation.

SUMMARY

1. The observations described in the present paper were made on connective-tissue cells derived from explants of rat-heart tissue.

2. Between 85 and 90 % of these cells emigrate from the explants before they begin to synthesize

deoxyribonucleic acid. The lag period preceding the onset of synthesis of deoxyribonucleic acid lasts about 20 hr.; for 12 hr. of this phase, the cells are outside the explant.

3. During the lag period there is continuous synthesis of both protein and ribonucleic acid. These two processes are prerequisites for the synthesis of deoxyribonucleic acid: if either is interfered with this latter synthesis does not take place.

4. The duration of the phase of synthesis of deoxyribonucleic acid varies widely. A median figure, obtained from several hundred cells, puts the beginning of this phase between 13 and 14 hr. before metaphase and its end between 3 and 4 hr. before metaphase; mitosis is thus immediately preceded by a period in which no synthesis of deoxyribonucleic acid occurs.

5. An analysis of the patterns of nuclear and nucleolar labelling in radioautographs of cells exposed to tritiated thymidine indicates that the synthesis of deoxyribonucleic acid is initiated at the nucleolus.

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A Substance from Animal Tissues which Induces Ketonuria

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Before insulin was generally available Petrén (1924) found that the addition of meat to the diet of diabetic patients could exacerbate or induce ketosis, whereas a diet containing a high proportion of fat reduced ketosis. Marks & Young (1939) made

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similar observations with dogs in which metahypophyseal diabetes had been induced. A reduction of ketonuria has also been observed when a high-fat diet is fed to alloxan-diabetic rats (Burn, Lewis & Kelsey, 1944; Bornstein & Nelson, 1949), and the exacerbating effect of dietary meat on the ketosis of alloxan-diabetic rats has been observed by Lazaris & Brzezinskaya (1946).

Marks & Young (1939) found also that if the meat in the diet of a diabetic dog was replaced by an amount of casein equivalent on the basis of total nitrogen, an existing ketonuria was greatly reduced. Moreover if, to a diet rich in fat, an aqueous extract of meat was added, ketonuria was greatly increased, though the residue left after hot-water extraction of meat did not greatly influence ketonuria when it was added to a high-fat diet.

The present investigation was undertaken to elucidate the nature of the ketonuria-stimulating substance observed by Marks & Young (1939) to be present in an extract of meat. A preliminary report of this investigation has already been published (Stewart & Young, 1952).

METHODS

Chemical reagents

2:4-Dinitrophenylhydrazine. This compound (Eastman Kodak Inc. or British Drug Houses Ltd.) was dissolved in 2N-HCl to give a 0.2% (w/v) solution. This solution was repeatedly extracted with purified CCl_4 until the extinction of the extract was less than 0.07 (d 1.4 cm. at 415 $m\mu$).

Carbon tetrachloride. This was washed for 24 hr. with running tap water and redistilled.

Calcium zinc- β -hydroxybutyrate. The DL sodium salt of this compound (Hopkin and Williams Ltd.) was converted into the free acid by acidification of a solution and continuous ether extraction. The residue after evaporation of the ether was treated according to the procedure described by Shaffer & Marriott (1913-14). The primary precipitate was crystallized from 95% ethanol and twice recrystallized from water. The product was dried *in vacuo* over P_2O_5 .

Periodate reagent. Sodium metaperiodate (6.40 g.) was dissolved in water and filtered through a glass filter (porosity 4) and made up to 100 ml. Calculated as the trihydrate, the material was 88% pure. Titration in acid indicated that the bulk of impurity behaved as iodate, a substance which is innocuous in periodate oxidation.

Methods of estimation

Ketone bodies. Urine was filtered immediately before analysis, samples of not more, and usually less, than 1 ml. being used. Urinary acetone plus acetoacetate was estimated by the method of Barkulis & Lehninger (1951) with the procedure for extraction described below. Urinary β -hydroxybutyric acid and total ketone bodies were estimated by the method of Greenberg & Lester (1944) modified in the following way. After oxidation had been effected under the conditions described by these authors, 3 ml. of the product was placed in a 25 ml. glass-stoppered cylinder. The residual dichromate was discharged with sulphite and the mixture treated successively with 5 ml. of 2:4-dinitrophenylhydrazine reagent and 10 ml. of purified CCl_4 . Extraction, the conditions for which are important, was carried out on a Townson and Mercer Ltd. laboratory shaker at 280-300 strokes/min. for 20 min. After the phases had separated, the supernatant was removed by suction and the solvent washed twice with 15 ml. of water by

vigorous shaking for 15 sec. The water was replaced with 10 ml. of 0.5N-NaOH and the solvent re-extracted for 15 min. on the shaker under the conditions described. The supernatant was removed and the extinction of the solvent estimated in a 1 cm. cell at 415 $m\mu$ by the Beckman model DU quartz spectrophotometer with a slit width of 0.1 mm. A standard estimation on calcium zinc β -hydroxybutyrate was carried through with each set of analyses.

The Rothera qualitative test for urinary ketone bodies was carried out according to the method described by Harrison (1947). Blood samples were prepared and analysed by the method of Greenberg & Lester (1944).

Urinary keto acids. These were determined by the direct procedure of Friedemann & Haugen (1943).

Blood sugar. This was estimated by the colorimetric method of Somogyi (1945).

Liver glycogen. This was estimated by the method of Good, Kramer & Somogyi (1933) on tissue quickly removed from an animal given a large fatal dose of Nembutal intraperitoneally.

Liver fat content. Slices from representative parts of the liver (total of about 300 mg. wet wt.) were weighed and dried to constant weight in tared containers for the determination of the ratio of wet wt./dry wt. The remainder of the liver was ground with twice its weight of anhydrous Na_2SO_4 and extracted in a Soxhlet apparatus for 4 hr. with methanol and for 8 hr. with ether. The combined extracts were evaporated to dryness *in vacuo*, the residue was taken up in light petroleum and filtered and the filtrate evaporated to dryness in a tared container which was weighed.

Spectrophotometry. Except where otherwise indicated, colorimetric estimations were carried out with a Unicam model SP. 350 spectrophotometer. Infrared-absorption spectra were obtained by means of a Perkin-Elmer double-beam instrument, model 21.

Animals

Hooded male rats weighing 150-160 g., of a strain bred in the Laboratory over a period of many years, were used in these experiments. In a few preliminary experiments similar results were obtained whether intact rats or alloxan-diabetic rats were used and all the observations subsequently made and recorded in the present communication were obtained in experiments with intact rats.

High-fat diet

In agreement with Burn *et al.* (1944) we found that the gradual addition of fat to the diet of the rat did not induce ketosis, whereas if the rat which had been eating normal food was suddenly transferred to a high-fat diet (e.g. one containing 70% of margarine) a substantial ketonuria developed. Nevertheless, if the animal continued to eat a suitable high-fat diet, the ketonuria steadily fell and in 15-30 days the urine became free from ketone bodies as judged by the Rothera test. In preliminary experiments it was found that a diet consisting of 70% of margarine and 30% of raw horse muscle produced a severe ketonuria, whereas one consisting of 70% of margarine, 6% of casein, salts and water did not. Accordingly a diet containing 70% of margarine and 6% of casein, together with 5% of salt mixture (Dunn Nutritional Laboratory salt mixture from British Drug Houses Ltd., London, based on that described by McCollum & Davis, 1915) and 19% of water,

was adopted as the standard high-fat diet in the present investigation. The calorie value of 100 g. of this diet, together with the caloric contribution of each principal component, is given in Table 1 together with data for the normal diet (diet 41, Bruce & Parkes, 1949) used for control animals.

The fat of the diet was Ministry of Food 'Special Margarine' consisting of about 83% of fat, 0.1% of protein and 15.5% of water, and containing 9.8% of linoleic acid and 0.2% of linolenic acid in addition to saturated even-number fatty acids from C_6 to C_{24} and some mono-unsaturated fatty acids. 'Low vitamin casein', supplied by Genatosan Ltd., Loughborough, Leics, was used in most instances. In a few experiments the casein was replaced by heat-coagulated dried horse muscle prepared by dropping minced fresh horse muscle into boiling water and maintaining it at 80–85° for 3–4 min., followed by straining through a sieve and air- and oven (100–105°)-drying. The dried residue was ground in a mechanical mortar into a soft fibrous powder.

The diet was prepared by adding the solid components to the margarine in a vessel which was warmed until the margarine was soft but not liquid. The water, salt mixture and casein were then blended into the margarine by vigorous beating and the mixture was allowed to set into a solid

paste, which was stored at 2° in tightly covered containers. Diet sufficient for a week to 10 days was prepared at one time and no tendency to develop rancidity was observed. For feeding to the rats the diet was packed into glazed earthenware dishes with overhanging edges. These in turn were placed in porcelain pots, a procedure which was found to minimize the scattering of food.

In addition to this food, with water *ad libitum*, a supplement of vitamins was given (Table 2). Vitamins A and D were present in more than adequate quantities in the margarine used. Parenteral administration of vitamins E and K had no effect on the weight curves or condition of the fat-fed animals and these vitamins were not always included in the diet. Inositol, in small and large doses, had no detectable effect on the weight curves or condition of the test animals and was omitted from the vitamin mixture because it was found to be antiketogenic (see also Wiebelhaus, Bethell & Lardy, 1947; Beatty & West, 1951). The other vitamins were prepared in two stock solutions, A and B (Table 2), which were mixed immediately before presentation to the rat. After a few days the supplement was readily taken by mouth. The composition of the mixture was so adjusted that approximately ten times the minimum requirements of the vitamins (Williams, Eakin, Beerstecher & Shive, 1950) were received daily. This large dose was considered desirable in order to prevent deficiencies arising from reduced intestinal absorption, or diminished intestinal synthesis, which might result from the high-fat content of the intestine. Nicotinamide, not normally required by the rat, was included because of the low-protein content of the diet.

The common B-group vitamins were obtained from Roche Products Ltd., Welwyn Garden City, Herts. Folic acid was obtained from Lederle Laboratories, London, and vitamin B_{12} was prepared by Glaxo Laboratories, Greenford, Middlesex.

Normal diet

This was diet 41 of Bruce & Parkes (1949), usually made into a paste with water.

Table 1. *Calorie contribution of the principal constituents of the high-fat and normal diets*

In the calculation of the composition of the high-fat diet no allowance has been made for the small amount of skim milk that may have been added to the margarine during manufacture. The carbohydrate calories of the high-fat diet are calculated from the glycerol content of the margarine.

Constituent	kcal./100 g. of diet		kcal. contribution (%)	
	High-fat diet	Normal diet	High-fat diet	Normal diet
Carbohydrate	22	198	4	67
Fat	499	41	93	14
Protein	16	58	3	19
Total	537	297	100	100

Table 2. *Water-soluble vitamin mixture for high-fat diet*

The total daily dose was dissolved to give 0.7 ml. of each of solutions A and B, which were mixed immediately before feeding.

Vitamin	Daily dose (mg.)
Solution A	
Thiamine	0.112
Riboflavin	0.140
Nicotinamide	0.700
Pantothenic acid	0.252
Pyridoxine	0.112
Biotin	0.014
Folic acid	0.238
Vitamin B_{12}	0.0007
Solution B	
p-Aminobenzoic acid	10.0
Choline	10.0

EXPERIMENTAL AND RESULTS

Effect of prolonged feeding of the high-fat diet on the normal rat

After a short time the rats ate the high-fat diet readily, for up to 2 years in some instances. From Table 3 the voluntary calorie intakes of rats receiving the high-fat diet and of those eating normal diet are seen to be very similar, but the mean daily gain in weight of the rats receiving the high-fat diet was subnormal (Table 3).

Most of the animals on the high-fat diet had greasy coats, and a loss of hair on the ventral parts of the trunk was sometimes observed. A few of the animals exhibited a peculiar deficiency syndrome, with lesions on the snout, trunk and tail, which was unresponsive to vitamin therapy but which usually cleared up if a small amount of carbohydrate was given to the animal. The rats which developed these lesions were not used in the studies on ketogenesis.

Table 3. *Calorie intakes of rats fed on the high-fat and normal diets*

Results given are mean values together with, in some instances, the standard errors of the means.

Diet	No. of rats	Period of observation (days)	Mean calorie intake (kcal./kg. of rat/day)	Mean gain in weight (g./day)
High-fat	5	21	439 ± 36.2	0.51
Normal	6	9	420 ± 16.5	5.0

Table 4. *Approximate water balance of rats on the high-fat and normal diets*

Diet	No. of rats	Period of observation (days)	Water drunk (ml./day)	Water present in food (ml./day)	Water calc. to be formed catabolically (ml./day)	Total intake of water (ml./day)	Urine excreted (ml./day)	Calc. inevitable water loss (ml./day)
High-fat	6	24	20.5	1.9	6.0	28.4	12.4	16.0
Normal	4	25	13.0	13.1	7.9	34.0	9.6	24.4

Table 5. *Analysis of blood and urine of non-fasting rats receiving the high-fat and normal diets*

Results given are the mean values together with the standard errors of the means. Figures in parentheses are the numbers of observations made.

Constituent	Unit of measurement	Value for rats on		Probability that difference is not significant
		High-fat diet	Normal diet	
(a) Blood				
1. Sugar	mg./100 ml.	85 ± 2.0 (7)	126 ± 17 (6)	<0.01
2. Ketone bodies	Equivalent to mg. of β -hydroxybutyric acid/100 ml.	10.50 ± 0.83 (16)	1.05 ± 0.26 (4)	<0.001
(b) Urine				
1. Keto acids	Equivalent to mg. of pyruvic acid/24 hr.	0.78 ± 0.21 (35)	1.80 ± 0.24 (22)	<0.001

Marks & Young (1939) remarked on the small volume of urine excreted by dogs consuming a high-fat diet, and Funk & Zefirow (1932) and Bornstein & Nelson (1949) made similar comments about the effects of a high-fat diet on the urine volume of the rat. We found (Table 4) that the water balance of the rats fed on our high-fat diet was not substantially abnormal. Since the observations on the experimental and control groups were not made simultaneously and since the ambient humidity was not controlled, the apparent greater urine excretion and smaller inevitable water loss of the rats receiving the high-fat diet is probably not of significance. In any case the urine excretion of rats on our high-fat diet was certainly not abnormally low.

The blood-sugar level of the rats on the high-fat diet was significantly subnormal (Table 5), whereas the blood ketone-body level appeared to be raised (Table 5). The urinary excretion of α -oxo acids was low in the rats fed on the high-fat diet (Table 5). The rats on the high-fat diet after a period of ketonuria usually excreted urine free from acetone and acetoacetic acid, as indicated by a negative Rothera test, the total daily ketone-body excretion (expressed as mg. of equivalent β -hydroxybutyric acid) being 0.47 ± 0.22 . A few of the animals on the

high-fat diet excreted urine which gave a positive Rothera test indefinitely, and these were excluded from those used in the studies on ketogenesis. It may be noted that when the casein of the high-fat diet was replaced by heat-coagulated dried horse meat, the mean ketone-body excretion rose more than 20-fold, with a possible slight diurnal variation in excretion, about 40%, of the total for 24 hr. being excreted between 10 a.m. and 10 p.m. When glucose, pyruvate and glycerol, and probably other anti-ketogenic substances, were fed to the rats receiving the high-fat diet in which casein had been replaced by dried horse meat, a sharp anti-ketogenic action was observed. This effect is illustrated for glycerol in Table 6.

Simultaneous estimation of blood and urinary ketone bodies of the rats receiving the standard high-fat diet, and of those given a similar diet in which the casein had been replaced by dried horse meat, yielded an opportunity of assessing the approximate renal threshold for ketone bodies in the rat receiving a high-fat diet. The results (Table 7) suggest a renal threshold of 20–30 mg./100 ml., a value similar to that observed for normal rats treated with pituitary extract by Shipley & Long (1938). Although other factors may alter the renal threshold for ketone bodies (Mirsky, 1938;

MacKay & Wick, 1939) the feeding of a high-fat diet is apparently not one of them.

The fat content of the livers of our rats receiving the high-fat diet was not very, though significantly, high (Table 8); the glycogen content was subnormal (Table 8). The size of the liver and its content of fat-free solids were within normal limits (Table 8).

Procedure for testing ketonuria-stimulating activity of meat extracts

The rats used for the detection of ketonuria-stimulating activity received the high-fat diet for at least 30 days before the test began and in many instances for much longer. The animals were kept in metabolism cages similar to those described by Ackroyd & Hopkins (1916). Urine was collected at the same time each day and analysed at once. No urinary preservatives were used.

Animals fed on the high-fat diet, whose urinary Rothera test had been negative for at least 10 days, were dosed by mouth with the material under test. If the substance to be

tested was a solid it was blended into the vitamin mixture; if it was a liquid it was given by stomach tube or dropwise into the back of the pharynx. It was customary to dose animals at the same time each day and in a single assay either two or three doses were given on successive days. Ketonuria usually appeared within 24 hr. of the completion of dosing with active material, but on some occasions did not appear for several days. Such a delay in response appeared to be a characteristic of particular animals and not of the test preparation, and may result from a delay in absorption from the gut.

Although this procedure is qualitative, experience has shown that the intensity and duration of the response may be used to give roughly quantitative information.

Preparation of ketonuria-stimulating extracts of meat

Fresh horse muscle was freed from excess of fat and fascia and minced. The mince was freeze-dried in batches of about 10 kg. and stored at 2°. The freeze-dried cake was broken into a coarse powder and extracted in 250 g.

Table 6. *Effect of feeding glycerol on ketosis of rats receiving a high-fat diet containing dried horse meat as the source of protein*

Day of experiment	Dose	Blood ketone body [mg. (as β -hydroxy- butyrate)/100 ml.] at 10.00 a.m.	Urinary excretion [mg. (as β -hydroxy- butyrate)/24 hr.]
1	—	45.1	34.6
3	—	40.8	49.8
6	400 mg. of glycerol per os at 10.15 a.m.	37.0	33.2
7	—	8.2	1.2
8	—	33.0	4.9
9	—	—	26.1
10	—	51.8	44.3

Table 7. *Blood and urine ketone-body contents for rats fed on a high-fat diet containing either casein or dried horse meat as the source of protein*

Dietary protein	Range of blood ketone-body contents [mg. (as β - hydroxybutyric acid)/100 ml.]	Number of observations	Urine ketone- body content [mg. (as β - hydroxybutyric acid)/24 hr.]
Casein	0.4-9	1	0.21
Casein	5.0-9.9	4	0.39
Casein	10.0-14.9	11	0.73 $\pm$ 0.66
Casein	15.0-19.9	7	1.00 $\pm$ 1.0
Casein	20.0-24.9	5	1.06 $\pm$ 0.47
Horse meat	30.0-34.9	1	12.5
Horse meat	35.0	6	63.5

Table 8. *Composition of liver tissue from rats receiving high-fat and normal diets*

Results given are the mean values together with the standard errors of the means. The figures in parentheses are the numbers of observations made.

Diet	Size of liver (g./100 g. of rat)	Amount in liver tissue (g./100 g. of liver tissue)			
		Water	Glycogen	Fat	Fat-free solids
High-fat	3.65 $\pm$ 0.55 (13)	69.1 $\pm$ 2.9 (8)	1.41 $\pm$ 0.54 (8)	7.26 $\pm$ 1.51 (13)	23.8 $\pm$ 3.9 (8)
Normal	3.47 $\pm$ 0.23 (8)	72.7 $\pm$ 0.5 (8)	4.5	4.80 $\pm$ 0.25 (8)	22.5 $\pm$ 0.75 (8)
Probability (<i>P</i>) that difference is not significant	>0.1	>0.1	—	<0.001	>0.1

portions with approx. 10 times its weight of butan-1-ol containing 10% (v/v) of water. Extraction was carried out with slow shaking for 12 hr., the solvent decanted and the extraction repeated four times. The extract contained the activity, and the residue, freed of solvent (dried extracted horse muscle), could be used as a source of protein in a non-ketogenic high-fat diet. The pooled extract was filtered, concentrated to about 250 ml. by vacuum-distillation at about 30°, washed twice with hot water to remove salts and filtered again. The filtrate, in a precipitating jar, was cooled to -10° overnight. This treatment converted the solvent into a semi-solid mass, which was thawed at 2°. The bulk of the brown supernatant was removed by suction and the tan-coloured residue was separated by centrifuging at 2°. This residue was taken up in warm butan-1-ol and cooled to 5°; an almost pure white solid deposited and the supernatant was discarded. This preparation was taken through the process of fractional crystallization shown in Fig. 1. Testing of fractions I, II (250 mg./day thrice) and IV (200 mg./day thrice) showed that fraction I possessed a trace of activity, II was inactive and IV produced a ketonuria which persisted for 14 days. Fraction III appeared to possess little or no activity. Tests on the concentrate from the butanol mother liquors were unsatisfactory because of difficulty in handling the viscous preparations and because of the anorexia they produced in test animals.

Some variation in ketonuria-stimulating activity was encountered among the 16 preparations of this sort obtained from different batches of horse meat. One preparation which had the expected melting point, and which was obtained in the expected yield, was inactive for no apparent reason. A typical response is shown in Fig. 2. In general 300–600 mg. of lipid was needed to induce a brisk response, and this amount of lipid corresponds to 500–1000 g. of fresh horse meat. Since the consumption by the rat of a diet consisting of 70% of margarine and 30% of raw horse meat and consumed at the rate of 10–15 g./day induces a similar ketonuria, it appears that the ketonuria-inducing activity of the lipid represents only a small proportion of the total activity of the raw meat.

Purification of the active substance. Some preparations were subjected to further purification by countercurrent distribution. This was carried out in graduated 150 ml. separating funnels lubricated with graphite. The composition of the solvent system selected (*n*-hexane–propan-1-ol–formamide, 40:40:10) was chosen as the result of preliminary investigations in which this system was found to yield the most suitable partition coefficient with undistributed material. The solvents, which had been freshly

Fig. 1. Procedure for fractional crystallization of crude muscle lipid.

Fig. 2. Effect of muscle lipid on the blood ketone-body level and the urinary excretion of ketone bodies. Each vertical arrow corresponds to a dose of 100 mg. of lipid.

redistilled, were equilibrated at room temperature, which was maintained during the distribution at $20^{\circ} \pm 1$. Solute (200 mg. when the volume of each phase was 25 ml.) was dissolved in the upper phase of funnel no. 1. The transfers (23) were carried out over a period of about 8 hr. On completion of the distribution the two-phase system in each of the separating funnels was treated with 50 ml. of glass-distilled water. The aqueous phase containing the formamide and some of the propanol was separated and the hexane fraction was washed with an additional 50 ml. of water. No solute could be demonstrated in these washings. The residual volume of solvent in each funnel was recorded. The weight curve for the distribution of the lipid was obtained by evaporating samples (2 ml. $\times$ 2) in tared glass shells (Craig, Hausmann, Ahrens & Harfenist, 1951) on a water bath, the solvent-free dishes being dried to constant weight. Colorimetric estimation of the ester bonds in the material was based on the formation of a coloured ferric hydroxamate, the procedure described by Hill (1946, 1947) being used. The colour was estimated at 520 m μ . Undistributed material was used in the construction of the calibration curve. Theoretical curves were calculated as described by Craig & Craig (1950). The total recovery from the distribution was 71%.

Chemical examination of ketonuria-stimulating extracts of meat

Saponification of 216.8 mg. of lipid was effected by heating with 20 ml. of ethanolic 90.12 mN-NaOH (CO_2 -free) on a boiling-water bath for 105 min. under conditions which excluded CO_2 . Titration of the residual alkali was carried out with standard 0.1N- H_2SO_4 in an atmosphere of N_2 . A control flask without lipid was put through the same procedure. The saponification mixture was evaporated to dryness on a boiling-water bath under a jet of N_2 , and drying was completed in a vacuum desiccator over CaCl_2 . The residue was taken up in warm water and transferred with washings to a small Kutscher-Studel extractor and acidified with dilute H_2SO_4 (final volume 50 ml.). The control material was treated in the same way. The precipitated fatty acid was removed by extraction with light petroleum

(b.p. 40 – 60°) and the residue transferred quantitatively with washings to a 100 ml. volumetric flask (final volume about 90 ml.). Periodate reagent (4 ml.) was added and the experimental and control flasks were made up to volume. Oxidation was allowed to proceed in the dark for 2 hr. at room temperature. Portions of the mixture (10 ml.) were then treated with 5 ml. of 0.5M-phosphate buffer, pH 7.4, and 5 ml. of 40% (w/v) KI and titrated with 0.1N-arsenite (see Bell & Greville, 1950) in order to estimate unused periodate. The end-point was sharpened by the use of polyvinyl alcohol as an indicator (Miller & Bracken, 1951). Titrations on blank and experimental solutions were carried out in duplicate.

The formic acid formed by periodate oxidation was estimated by titration after steam-distillation in the apparatus of Markham (1942). The periodate oxidation mixture (15 ml.) was treated with 0.5 ml. of redistilled, acid-free, ethylene glycol to destroy the residual periodate and the mixture was made up to 25 ml. with 10% (w/v) solution of KHSO_4 . Portions of this solution (5 ml.) were steam-distilled until 65 ml. of distillate had been collected. Titration was carried out under N_2 with 9.37 mN-NaOH dispensed from a Conway microburette, phenol red being used as indicator. The titration value for a blank experiment was subtracted from the result. A standard solution of formic acid gave a value of 98.3–98.6% of that expected, by this method.

The formaldehyde formed by periodate oxidation was estimated by a method adapted from that of Bell & Greville (1950). The periodate oxidation mixture (10 ml.) was treated in a 50 ml. beaker with 3 ml. of N-HCl and 5 ml. of 1.2M-potassium arsenite. The solution was then acidified with sufficient HCl to produce a yellow colour with bromocresol green (3 drops of 0.1%, w/v, in ethanol). The colour was then adjusted with M-sodium acetate to a blue-green. Dimedone (250 mg. in 2 ml. of ethanol) was added to the mixture, which was digested for 2 hr. on a boiling-water bath and then allowed to stand for $3\frac{1}{2}$ hr. at room temperature. The deposited derivative was collected in a tared sintered-glass thimble, washed with 75 ml. of water, dried *in vacuo* overnight over P_2O_5 and weighed.

Chemical properties of the ketonuria-stimulating substance

Composition. The most active preparation (fraction IV) was pure white, crystalline, insoluble in water but soluble in lower alcohols (more so in butanol than in methanol), acetone, warm (40°) acetic acid, light petroleum, benzene and ether. The melting point was relatively sharp and ranged from 52° to 54° (uncorrected) in different preparations. Sodium fusion revealed no trace of nitrogen, sulphur or halogen and a spark spectrum (Johnson Matthey and Co., London) failed to reveal significant quantities of metallic compounds. Microanalysis (Weiler and Strauss, Oxford) gave C, 74.9–76.5; H, 11.7–12.8. Calc. for tristearin C, 76.2; H, 13.0; for tripalmitin C, 75.4; H, 12.9%.

With the cooling-curve method of Reilly & Rae (1948) a molecular weight of 650 ± 50 was obtained. Saponification of 100 or 200 mg. of the lipid gave

a saponification number of 208.3, which corresponds to a molecular weight of 808.3 if a completely esterified trihydric alcohol is assumed. If the alcohol is glycerol then calculation shows that 11.42 mg. is liberated from 100 mg. of lipid.

The saponification mixture was freed from fatty acids by acidification with dilute H_2SO_4 and extraction with light petroleum, and treated under controlled conditions with sodium periodate. The periodate consumption and the formaldehyde and formic acid production were estimated in order to estimate glycerol set free. When the formic acid value was corrected for the small amount which was not volatile in steam under standard conditions the formaldehyde/formic acid ratio was 2.008, almost precisely that expected for glycerol. In Table 9 the glycerol content of the lipid, as determined by a number of methods, is shown. The small difference between that deduced from the saponification number and those based on the other

methods of analysis is probably due to the presence of a trace of free acid in the lipid. The presence of a trace of acid could be demonstrated by extraction with dilute $NaHCO_3$ or by steam-distillation.

The presence in the saponification mixture of no polyhydric alcohol other than glycerol was demonstrated by the paper-chromatographic method of Hough (1950).

Since the iodine number of several preparations was 6-8 the fatty acids present appeared to be essentially saturated.

Spectroscopic examination. No absorption of light of wavelength above $240\text{ m}\mu$ was observed for a light-petroleum solution of lipid (1 mg./ml.). The infrared-absorption spectra of two typical preparations are shown in Fig. 3. Comparison of the spectra with the data of Shreve, Heether, Knight & Swern (1950, 1951a, b) shows that the general shape of the spectrum corresponds with that reported for saturated triglycerides, but the presence of additional bands, at 10.86 , 11.25 , 11.93 - 11.99 and $13.77\text{ }\mu$, suggests the existence of an oxirane (ethylene oxide) ring, a suggestion which is supported by the presence of a complex of small peaks at 7.09 , 7.27 , 7.69 , 7.95 , 8.21 and $8.37\text{ }\mu$. A band at $10.31\text{ }\mu$ is generally considered to be related to a *trans* double bond.

Homogeneity. The homogeneity of a triglyceride is difficult to establish unequivocally. Counter-current distribution was applied to the problem

Table 9. Glycerol content of muscle lipid determined by different methods

Method	Glycerol from 100 mg. of lipid (mg.)
Saponification	11.42
Periodate consumption	11.23
Formic acid (corrected)	11.24
Formaldehyde	11.26

Fig. 3. Infrared-absorption spectra of two samples of horse-muscle lipid. The upper curve in each case was derived from a solid film of lipid and the lower comes from a Nujol (liquid paraffin) mull.

but a difficulty in the interpretation of the results arose from a change in the volume of the phases with changing concentration of solute (see also Olley, 1953). In Fig. 4 the distribution of the horse-muscle lipid in *n*-hexane-propan-1-ol-formamide is shown. Gravimetric and colorimetric estimation of the solute in each separating funnel were both made with the object of demonstrating that the mass peak corresponds to a single type of chemical species. If preparations at the peak of the curve consisted of a mixture of hydrocarbon, glycerol ether and glyceride, the weight curve would not correspond to the curve based on ester-group analysis. The curves indicate that the principal component is a single type of chemical species.

Since the volumes of upper and lower phase did not remain constant throughout the distribution, the conventional method of binomial analysis could not be applied. If it is assumed that the partition coefficient does not vary during the course of the distribution and a record is kept of volume changes in upper and lower phases (this can easily be done by using graduated separating funnels), it is possible to apply a correction to each term of the binomial after each transfer for the altered volume relationships, and in this way a 'corrected' theoretical curve can be obtained. The greatest volume change was found to occur between the sixth and tenth transfers, and thereafter the conditions in the first twelve funnels remained approximately constant. To calculate the theoretical curve therefore a normal distribution with an empirical selected partition coefficient was cal-

culated for the first six transfers. Thereafter the ratio of upper to lower phase prevailing at the end of the distribution (after twenty-four transfers) was introduced to permit the calculation of the absolute amount transferred with each movement of solvent. The procedure involves the calculation of 189 terms with each selected partition coefficient. In Fig. 5 the results of the calculation are shown and it can be seen that when a partition coefficient of 0.45 is used a curve closely corresponding to the observed curve is obtained. The skewing of the experimental curve does not therefore necessarily indicate that the material distributed was a mixture.

DISCUSSION

The evidence presented suggests that the ketogenic material isolated from horse muscle is a triglyceride. Since it is active in small doses in an animal receiving a large amount of triglyceride in its diet, the extract cannot be ketogenic solely or mainly by virtue of its ability to give rise to ketone bodies. Some fraction of the glyceride, or a substance contaminating, must be an active ketogenic material. A similar material can be obtained from calf intestinal mucosa (Stewart & Young, 1952). Although a crucial test of homogeneity has not been found, such evidence as is available suggests

Fig. 4. Countercurrent distribution of muscle lipid in *n*-hexane-propan-1-ol-formamide (40:40:10) system; 200 mg. of solute was distributed in the system. The volumes of the upper and lower phases at the beginning of the distribution were 25 ml. Δ — Δ , Distribution by weight; $\bullet$ — $\bullet$, distribution of ester bonds on corresponding ordinate scale.

Fig. 5. Calculated theoretical curves, corrected for changes in phase volume, on the basis of three different assumed values of the partition coefficient (K). —, Experimentally determined curve; ---, K 0.35; - · -, K 0.45; · · ·, K 0.50.

that our best preparations are unlikely to be contaminated with material other than glycerides. Glycerol is highly anti-ketogenic (cf. Table 9), and the ketopoietic properties of the glyceride are almost certainly to be attributed to the fatty acid part of the substance.

Consideration of the results of microanalysis and of the infrared-absorption spectrum suggests that one or more oxygen atoms may be attached to the fatty acid chains. The reaction of 2:4-dinitrophenylhydrazine with the lipid reported earlier (Stewart & Young, 1952) may have been with an oxygen atom of this sort, or possibly with an ester group to form a hydrazide. The dinitrophenylhydrazine derivative was difficult to purify and its preparation, like that of the benzylidene derivative, has not been consistently reproducible.

The physiological activity of partially oxidized fatty acids has not been studied in detail. In most of the reported experiments on the feeding of rancid fats the effects observed were referable to destruction of the vitamins in the diet. In our experiments there was no evidence of known vitamin deficiency. Kaunitz (1953) has studied the effect of the administration of rancid fats to rats under conditions which he believes precludes the complications which result from vitamin deficiency. He showed that these materials were absorbed from the gut and that they interfere with growth but no evidence was provided to elucidate the nature of the metabolic disturbance produced by the rancid fat in his experiments.

SUMMARY

1. Evidence has been provided for the existence of a ketonuria-stimulating substance which can be extracted from horse muscle.

2. An animal suitable for the detection of such a ketopoietic material is the normal rat given a diet rich in fat. Some of the physiological characteristics of this test animal have been described.

3. A method for the isolation of the ketopoietic substance from horse muscle has been evolved and evidence has been provided which suggests that the isolated material is homogeneous.

4. Chemical examination indicates that the isolated material is a triglyceride, and some evidence has been provided that a fatty acid chain in the glyceride is in a partially oxidized state.

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