TABLE V. Influence of Estrous Cycle on Collagen Synthesis by Rat Uterine Slices.

Vaginal smear	No. of animal	Wt of uterus (g)	mg collagen / g of uterus	Total collagen (mg)	No. of sample	Specific activity (cpm/mg)* Collagen	Hydroxy-lysine	Lysine
A Epithelial cells only	3	.90 ±.08† A=B<.05‡	71 ± 1	64 ± 5 A=B<.05	6	51 ± 4 A=C<.05	416 ± 64	1790 ± 108
B Cornified cells only	4	.54 ±.08	71 ± 6	38 ± 6	4	43 ± 5	393 ± 61	1440 ± 123
C Leucocytes and epithelial cells	3	.67 ±.10	69 ± 5	48 ±10	4	39 ± 3	333 ± 50	1480 ± 123

* See legend, Table I.
† Mean ± standard error(8).
‡ P values relating to age differences, when within 5% level of confidence, are listed. Statistical calculations are made according to Bailey(8).

is emphasized by the observations of Harkness(7), that uterine collagen increases during pregnancy.

Summary. 1. Factors affecting collagen synthesis *in vitro* have been studied in uterine slice systems of the rat. 2. The results agree well with those of our previous report concerned with collagen synthesis in sponge biopsy connective tissue. Elimination of NaCl from the incubation medium was accompanied by a more striking decrease in collagen synthesis than followed the removal of any other constituent. Elimination of KH_2PO_4 from the incubation medium caused a significant decrease in collagen synthesis. Maximal collagen synthesis occurred when the starting pH was 8.4 and the gas in the incubation vessels was composed of 95% oxygen and 5% carbon dioxide. 3. Optimum temperature for keeping tissue prior to incubation was 0°. Optimum incubation temperature was 37°. 4. Collagen synthesis reached a peak in uterine slices obtained from animals at the height of follicular hormonal activity.

The authors gratefully acknowledge suggestions and advice of Dr. C. R. Treadwell.

1. Kao, K. Y. T., Hilker, D. M., McGavack, T. H., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 121.
2. Kao, K. Y. T., Lu, S. D. C., Hitt, W., McGavack, T. H., *ibid.*, 1962, v109, 4.
3. Kao, K. Y. T., Hitt, W. E., Dawson, R. L., McGavack, T. H., *ibid.*, 1963, v113, 762.
4. Scharpenseel, H. W., Wolf, G. F., *Tierernähr. Futtermittelk,* 1959, v14, 347.
5. Fujimoto, D., Tamiya, N., *Biochem. J.,* 1962, v84, 333.
6. Prockop, D. J., Kaplon, A., Udenfriend, S., *Biochem. & Biophys. Res. Communs.,* 1962, v9, 162.
7. Harkness, M. L. R., Harkness, R. D., *J. Physiol.,* 1956, v132, 492.
8. Bailey, N. T. J., *Statistical Methods in Biology,* John Wiley & Sons, Inc., New York, 1959, p169, 172.

Received September 25, 1963. P.S.E.B.M., 1964, v115.

A Fat Mobilizing and Anorectic Substance in the Urine of Fasting Rats.* (28931)

J. A. F. STEVENSON, B. M. BOX AND A. J. SZLAVKO
(Introduced by J. R. Beaton)
Department of Physiology, University of Western Ontario, London, Ont., Canada

Weil and Stetten(1) reported an increase in the liver lipid content of mice receiving extracts of urine obtained from fasted rabbits. Chalmers *et al*(2) reported isolation of a similar fraction from urine of fasted humans, using the same procedure. In the intact mouse, subcutaneous injections of this extract caused a transient hypoglycemia, ke-

* Supported by a grant from Medical Research Council of Canada.

tonemia, hyperlipemia, and increased mobilization and catabolism of fat, with depletion of body fat stores. There was also an increase in blood lipids, including total lipids, phospholipids and free fatty acids, although larger relative doses were necessary to produce unequivocal effects. No change in appetite and food intake accompanied the increased fat catabolism and the decrease noted in body weight. *In vitro* studies showed that a concentration of less than 1 μg/ml of the active extract was effective in releasing free fatty acids from rat epididymal fat pads. Further details of the characteristics of this fat mobilizing substance (FMS) have been reported by the same authors(3,4) and they postulated that FMS mobilizes fatty acids from adipose tissue by directly augmenting the lipolytic reaction. FMS activity did not appear in the urine of fasted hypophysectomized man or goat and they concluded that the pituitary (or adjacent hypothalamus) was necessary for its production(3).

Our interest in the hypothalamic control of food intake and hypothalamic obesity prompted us to try similar experiments in the rat.

Methods. I. *Extraction.* Animals to be used for urine collections were placed in special metabolism cages (Acme Metal Products Inc.) and urine collected under toluene. Preparation of the extract from 24-hour combined urine collections was as follows: 1. precipitation with alcoholic benzoic acid at pH 5.3; 2. washing with ethanol; 3. extraction of the residue with 0.5% sodium carbonate; 4. reprecipitation with ethanol at pH 5.3; 5. re-extracting residue with 0.5% sodium carbonate; 6. drying supernatant in vacuum desiccator to calculate yield of FMS and 7. redissolving in distilled water for storing at —10°C. Before bioassaying or injection, extract was adjusted to pH 7.4 with 0.1 N HCl.

II. *Bioassay.* Adipose tissue samples weighing approximately 50 mg were excised from the epididymal fat pads of male Sprague-Dawley rats under sodium pentobarbital anesthesia. Tissue samples were weighed accurately on an analytical balance and then placed in 10 ml Erlenmeyer flasks containing 1 ml of incubation medium (4% bovine serum albumin in Krebs-Ringer bicarbonate buffer at pH 7.3-7.4 or pooled rat plasma).

After addition of the extraction solution (containing 20 μg of FMS) or an equal volume of distilled water for the control, free fatty acids were determined on 0.4 ml aliquots of the incubation medium by the method of Dole(5). The remaining medium and fat pad were incubated for 3 hours at 36°C in a Dubnoff Metabolic Shaking Incubator using nitrogen as the gas phase. After incubation, free fatty acids were again determined and their release from the adipose tissue by the extract expressed in micromoles per 100 mg of tissue.

III. *Chemical properties.* One dimensional paper chromatography with butanol:water: acetic acid (4:5:1) solvent was carried out on the extract after hydrolysis in 6 N HCl for 20 hours at 100°C. Total nitrogen after digestion in sulfuric acid was determined colorimetrically using the Nessler reaction. Carbohydrate was determined by the anthrone method (6).

IV. *Biological effects.* Active extracts were injected into normal and hypothalamic, hyperphagic rats of the Sprague-Dawley strain. Body weights, food and water intakes were measured using a Shadograph scale. The animals were housed in individual cages at an environmental temperature of 23° ± 1°C and animals were fed a semi-synthetic diet providing 5.5 calories/g having 21% of the calories as carbohydrate, 20% as protein and 59% as fat. Food and water were allowed *ad libitum.* Blood glucose was determined by a micro-adaptation of the method of Somogyi (7) using 0.02 ml of whole blood. Blood ketones were measured as acetone and acetoacetate by the method of Pawan(8). Total liver fatty acids were determined titrimetrically on an acidified petroleum ether extract using 0.02 N KOH. Plasma free fatty acids were determined by the method of Dole(5). Resting oxygen consumption was measured just prior to injection and again 4 hours post-injection using the procedure of Haldi *et al* (9).

V. *Hypothalamic hyperphagic animals.* Hyperphagic rats were prepared by making bilateral electrolytic lesions in the ventromedial

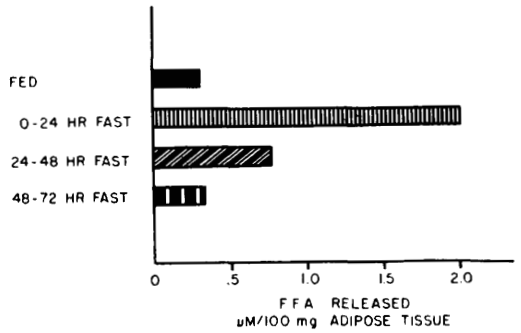


FIG. 1. Relative activity of FMS extract collected from rats in the fed state, during 0-24 hr fast, 24-48 hr fast, and 48-72 hr fast. Activity is measured as μM of free fatty acids released from 100 mg of epididymal adipose tissue.

nucleus of the hypothalamus using a Horsley-Clarke stereotaxic apparatus.

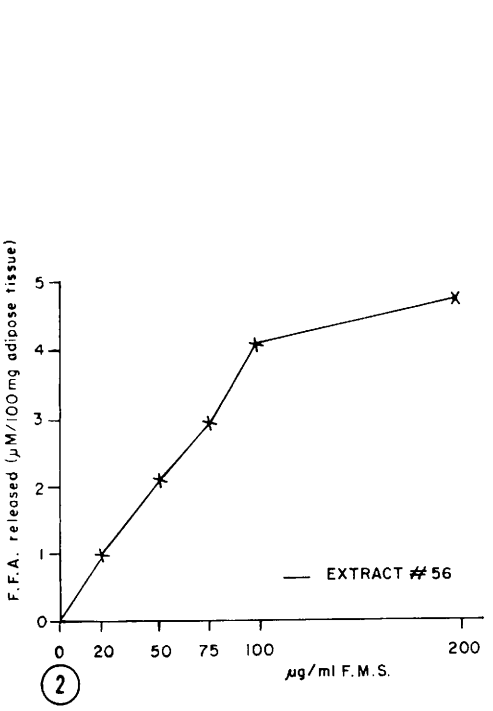
Results. Bioassay. To determine the relative activity of FMS during various stages of fast we used combined 24-hour urine collections from 12 male Sprague-Dawley rats 7 weeks of age. Pooled rat plasma was used as the incubation medium. An initial collection was made on Day 1 when the animals were fed their standard chow diet. The animals were then fasted for 3 days with water provided *ad libitum* and separate urine collections made for each 24-hour period. The greatest activity was obtained at the beginning of the fast (0-24 hr)—2.061 μM of free fatty acids were released per 100 mg of adipose tissue (Fig. 1). There was some activity in the urine excreted during the fed state (0.314 μM of free fatty acids released per 100 mg tissue); this was approximately equal to the activity of the extract obtained during 48-72 hours of fasting (0.325 μM of free fatty acids/100 mg tissue). Hypothalamic obese rats also excrete FMS. Two male hypothalamic obese rats (each weighing about 600 g) previously fed a high fat diet were fasted for 24 hours and the urine collected. The activity of the FMS extract was 2.597 μM of free fatty acids released per 100 mg of adipose tissue.

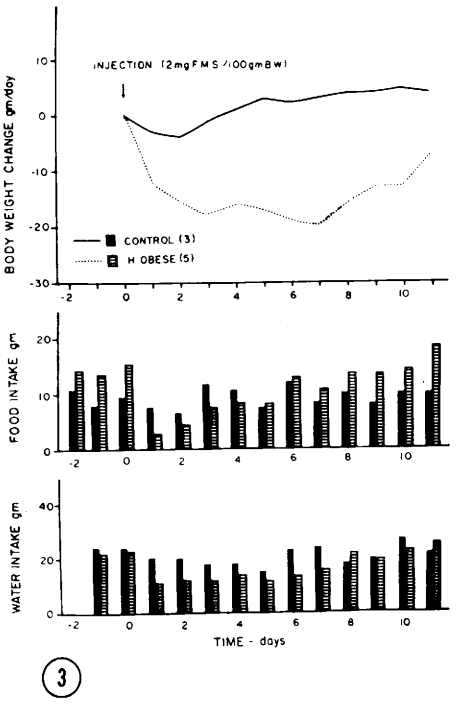
To obtain a large quantity of extract, 24 male Sprague-Dawley rats were fed a chow diet *ad libitum* on alternate days. Separate collections were made in the fed and fasted states over a period of 4 weeks. The extracts of the urine collected in the fasted state were combined. The dose-response curve for this extract is shown in Fig. 2.

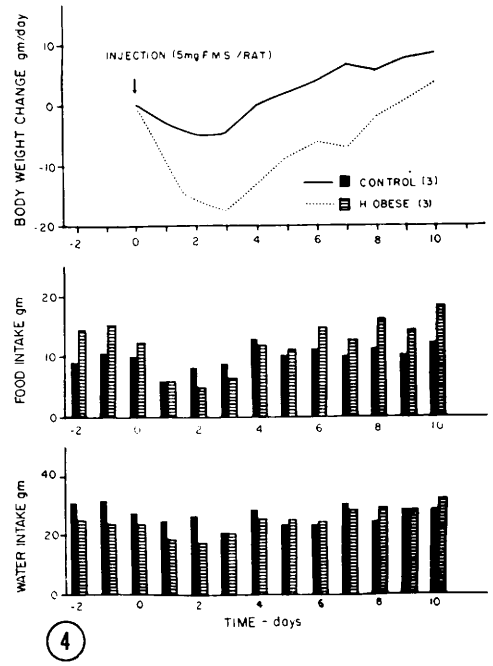
Chemical properties. Preliminary analyses by paper chromatography after acid hydrolysis of the rat FMS extract indicate that it is a polypeptide composed of histidine, phenylalanine, leucine, serine, cystine, aspartic acid and alanine, with traces of 2 other amino acids. The extract obtained from fasted rats contained about 10% nitrogen and 6% carbohydrate. These results are very similar to those reported by Chalmers *et al*(3) from their analysis of human FMS.

Effect on body weight and food and water intake. A decrease in body weight which was paralleled by a decrease in food intake was observed in both intact and hypothalamic obese rats injected with FMS. Water intakes decreased to a lesser degree. As a consequence, water/food ratios showed an increase for several days following injection of FMS. The hypothalamic obese rats were more "sensitive" to FMS than were normals (Fig. 3, 4). At the first injection (Fig. 3) all animals received 2 mg of FMS per 100 g body weight; control rats weighed an average of 287 g and hypothalamic obese rats weighed 582 g. At the second injection, 5 weeks later, each animal received 5 mg FMS per rat (Fig. 4), at which time control rats weighed 297 g and hypothalamic obese rats 607 g. The hypothalamic obese rats showed a greater drop in food intake and body weight in response to FMS than their controls whether it was administered on an absolute basis or in relation to body weight.

Other biological effects. Chalmers *et al*(3) reported a transient hypoglycemia in the intact mouse after subcutaneous injection of FMS. Using 18 male Sprague-Dawley rats approximately 3 months old we injected (s.c.) 2 mg of rat FMS into 6 animals, 4 mg into 6 and the vehicle into the remaining 6. Tail blood samples were taken at hourly intervals for measurement of blood glucose. The results shown in Fig. 5 demonstrate a transient hypoglycemia from both doses of FMS with the lowest glucose values obtained 3 hours post-injection.







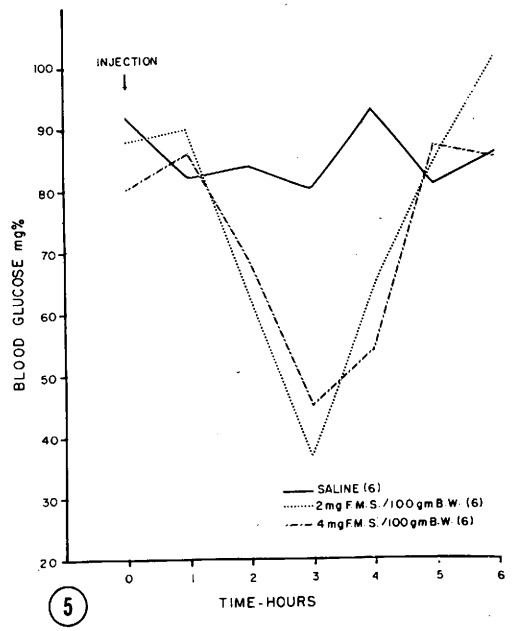


FIG. 2. Dose response curve for FMS Extract #56 was obtained from rats in a fasted state which were fasted on alternate days.

FIG. 3. Effects of injections of FMS (2 mg/100 g of body weight) on body weight, food intake and water intake of control and hypothalamic obese female rats. At time of injection control rats weighed an average of 287 g and hypothalamic obese rats an average of 582 g.

FIG. 4. The effect of injections of FMS (5 mg per rat) on the body weight, food intake and water intake of control and hypothalamic obese female rats. At the time of injection, the control rats weighed an average of 297 g and the hypothalamic rats weighed an average of 607 g. These injections were carried out 5 weeks after the injections, results of which are shown in Figure 3.

FIG. 5. Effect of FMS injections on blood sugar levels of fed rats.

TABLE I. Effect of a Single Injection of FMS on Blood Sugar, Blood Ketones, Plasma Free Fatty Acids and Liver Fatty Acids in the Normal Fed Rat.

	Controls		2 mg FMS	
Time	0 hr	4 hr	0 hr	4 hr
No. of rats	(2)	(3)	(3)	(3)
Blood sugar (mg %)	99 ± .0	101 ± 12.6*	86 ± 9.9	44 ± 6.5
		>.1†		<.05
Blood ketones (mg/100 ml)	1.2 ± .10	.8 ± .43	1.0 ± .62	2.3 ± .97
		>.1		>.1
Plasma free fatty acid (μeq/l)	—	510 ± 37.8	531 ± 11.3	625 ± 37.8
				<.05
Liver fatty acids (%)	2.3 ± .58	1.9 ± .11	1.9 ± .00	3.2 ± .33
		>.1		<.001

* Mean ± S.E.M. † P value.

A group of 11 male rats was used to determine the effects of FMS on blood ketones, plasma free fatty acids and liver fatty acids. The experimental animals received 2 mg of FMS/100 g of body weight, and controls an equal volume of the vehicle. Half of each group was killed immediately after injection by decapitation; the remaining animals were killed 4 hours later. Blood samples were collected in heparinized tubes and one lobe of the liver was removed. There was a decrease in blood sugar, demonstrated previously, but the increase in blood ketones was only suggestive (Table I). There was a definite increase in plasma free fatty acids and liver fatty acids.

The resting metabolic rate after injection of FMS was measured on a group of 3 hypothalamic obese female rats and their 3 controls before and 4 hours after injection of 5 mg FMS per rat (s.c.). At the time of test, the obese animals weighed approximately 600 g and the controls 250 g. There was a suggestive decrease in metabolic rate in both groups although this was not statistically significant (Table II).

Discussion. In agreement with the observations of Chalmers *et al*(3) on the effects in the mouse of an extract from the urine of fasting man, we found that a similar extract from the urine of fasting rats when injected into intact rats produced a weight loss, transient hypoglycemia, hyperlipemia, and an increase in fatty acid content of liver. Contrary to the findings of Chalmers *et al*, however, a reduction in food intake, which appeared to be sufficient to explain the weight loss, invariably occurred. This discrepancy may be due to a species difference in the extract or in the test animal. The anorectic effect of rat FMS is, like that of amphetamine(10) and phenmetrazine(11), much more pronounced in the hypothalamic hyperphagic than in the normal rat. This hypersensitivity of the hypothalamic hyperphagic rat to anorectic agents has not been explained. The present observation emphasizes that lesions of the ventromedial region do not destroy all of the inhibitory systems of feeding.

It is realized that there is an apparent paradox in these observations. Although the rat FMS appears to be anorectic, it is excreted, and presumably produced, in the largest quantity when the animal gives evidence of being most hungry, *i.e.*, during the first 24 hours of starvation. The anorectic action of the FMS could be due either to a direct action on the neural systems regulating food intake or to an indirect effect on these

TABLE II. Effect of a Single Injection of FMS on Resting Oxygen Consumption of Normal and Hypothalamic Obese Rats.

	Metabolic rate ($ml\ O_2/min/kg\ ^{3}/_{4}$)	
	Controls (3)	H. obese (3)
0 hr	14.2 ± .83*	10.6 ± .49
4 hr post-inj	12.1 ± .54	8.6 ± .60
P value	>.1	>.1

* Mean ± S.E.M.

through the increase in the level of circulating FFA. The latter effect would fit the lipostatic hypothesis for regulation of food intake(12) rather than the glucostatic(13) or thermostatic(14) hypotheses. The present preliminary evidence that, if anything, injection of this substance causes a reduction in oxygen consumption, argues against a thermostatic mechanism and the hypoglycemia against a glucostatic mechanism being involved in this particular inhibition of food intake. It is intriguing, however, to speculate that there may be hormones or hormone metabolites that directly effect the nervous regulation of food intake. The origin, characteristics, actions and factors affecting the secretion of this substance in the rat are being investigated.

Summary. A substance has been extracted from the urine of fasting rats that causes an increase in release of free fatty acids from rat epididymal fat pads *in vitro* and in the serum FFA of the rat *in vivo*. This fat mobilizing substance (FMS) is similar in chemical composition (containing at least 7 amino acids and some carbohydrate) and in biological activity (causing a hypoglycemia and body weight loss in addition to lipolysis) to FMS extracted from urine of fasting man by others. It does, however, cause a significant decrease of food intake in the rat, particularly in the hypothalamic hyperphagic animal.

The authors wish to acknowledge the helpful advice of Dr. W. C. C. McMurray and Dr. J. R. Beaton.

1. Weil, R., Stetten, Jr., *J. Biol. Chem.*, 1947, v168, 129.
2. Chalmers, T. M., Kekwick, A., Pawan, G. L. S., Smith, I., *Lancet*, 1958, v1, 866.
3. Chalmers, T. M., Pawan, G. L. S., Kekwick, A., *ibid.*, 1960, v2, 6.
4. Cahill, G. F., Jr., Pawan, G. L. S., Chalmers, T. M., *Endocrinology*, 1961, v69, 648.
5. Dole, V. P., *J. Clin. Invest.*, 1956, v35, 150.
6. Morris, D. L., *Science*, 1943, v107, 254.
7. Somogyi, M., *J. Biol. Chem.*, 1952, v195, 19.
8. Pawan, G. L. S., *Biochem. J.*, 1958, v68, 33.
9. Haldi, J., Wynn, W., Breding, H., *J. Appl. Physiol.*, 1961, v16, 923.
10. Stowe, E. R., Jr., Miller, A. T., Jr., *Experientia*, 1957, v13, 114.
11. Stevenson, J. A. F., Montemurro, D. G., Bernardis, L. L., *Fed. Proc.*, 1958, v17, 156.
12. Kennedy, G. C., *Proc. Roy. Soc.*, 1953, vB140, 528.
13. Mayer, J., *Ann. N. Y. Acad. Sci.*, 1955, v63, 15.
14. Brobeck, J. R., *Recent Prog. Horm. Research*, 1960, v16, 439.

Received September 26, 1963. P.S.E.B.M., 1964, v115.

Effect of High Linoleic Acid and Antioxidant Deficient Diet on Blood Serum Proteins in Chicks.* (28932)

LOUIS L. TUREEN, JOHN CONOMY AND DANIEL K. LEE
(Introduced by Theodore Cooper)
Max and Anna Tureen Neurology Research Laboratory, and Department of Neurology and Psychiatry, St. Louis University School of Medicine, St. Louis, Mo.

Chickens fed diets high in linoleic acid and deficient in Vit. E develop an encephalomalacia which is accompanied by abnormal electroencephalic tracings(1). The mechanism of the production of lesions in the brain in antioxidant deficiency states in chickens is unknown. Altered capillary transfer due to antioxidant deficiency has been considered a cause of the changes(3,4). Whether this is due to incorporation of abnormal metabolic products into the cell membrane, or to alterations in blood serum osmolality(5) are questions still under discussion. Changes in blood serum proteins in certain exudative diatheses occurring in selenium and antioxidant deficient diets support the view of others that changes in capillary permeability are respon-

* This study was supported by grant from Nat. Inst. of Neurological Diseases and Blindness.