Liver Ketogenesis V. *In vitro* Acetoacetate Production and Utilization in Rats Fed High Fat-high Casein Diet.* (27404)

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It has long been known that the type of food ingested affects the extent of ketonuria during consumption of any particular diet, and thereafter during a period of fasting. We have reported that high fat feeding(1) and hyperthyroidism(2) increase ketogenesis in surviving liver slices from different substrates. Fasting ketonuria in these rats was greater and was further increased by prolonged feeding of a high fat diet. High fat feeding and hyperthyroidism were also accompanied by various derangements in carbohydrate and lipid metabolism giving rise to hyperglycemia, fatty livers and hypertrophy of tissues *e.g.*, liver, kidneys and adrenals. Deuel *et al.*(3) observed increased ketonuria in human subjects maintained on high fat-high-protein diets. The association of ketosis with diabetes is well established and significant. The diabetogenic action of thyroid has been discussed by Houssay(4). With the above findings in mind, experiments were done with special attention to the composition of different diets and to period of feeding, to study the influence of high fat-high casein diet alone or with thyroid and thiouracil on liver ketogenesis and extra-hepatic utilization of ketone bodies.

Methods. Male albino rats, 125-150 g body weight and growing steadily on a standard diet, were divided into 7 groups of 12 rats each. These rats were fed different diets *ad libitum* along with the vitamin and salt mixtures(5). The composition of the diet and vitamin mixture is shown in Table II. We have reported(1) the differences in metabolism of rats while fed high fat diet for 7 and 19 weeks respectively. Therefore, 2 periods of feeding were chosen in these experiments, to determine the effect of prolonged feeding of high fat-high casein diet alone or in combination with low doses (.15%, Group CFTa) of thyroid, which develops hyperthyroidism gradually as compared with the rapid onset induced by feeding desiccated thyroid in higher amounts (.45%, Group CFT). Thiouracil (.3% of the diet by wt) was given to rats in Group CFU.

Animals were weighed at every weekend and their urine analysed for ketone bodies and sugar.

On the completion of feeding period as shown in Table II, animals were fasted for 20 hr, then sacrificed by stunning and decapitation. Blood and required tissues were collected immediately.

Detailed methods for tissue preparation and chemical estimations have been published (2).

Results and discussion. Observations on body weight and food consumption of rats in different groups (Table I) indicate that high casein feeding (Group HC) somewhat retards the normal growth of rats, as previously observed by Bosshardt *et al.*(6). Rapid onset of hyperthyroidism (Group CFT) reduces body weights appreciably even though the food consumption of these rats is one and a half times more than in normal rats. But, when hyperthyroidism is induced gradually, rats maintain better weights. Although hypothyroid rats eat less, their body weights are nearer to normal. These changes in body weights are probably due to the effect of thyroid and thiouracil on general metabolism and

TABLE I. Weekly Body Weight Gain, g/100 g Body Wt of Rats in Different Groups.

Group	Period in wk 1	2	3	4	5	8	10	Avg food intake, g/rat/day
N	10	16.5	26	34	—	—	—	12
HC	8	14	19	24	—	—	—	11
CF	7.5	13	20	26	—	—	—	8
CFa	8	15	21	27	—	55	74	8
CFT	5	8	5.5	2.5	—	—	—	11.5
CFTa	7	13	17	20	—	24	—	10.5
CFU	8	15.5	22	27.5	34	—	—	6

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TABLE II. Composition of Diets, %.*

Group	No. of rats	Feeding period in wk	Starch	Casein (E. Merk)	Ground-nut oil	Salt mixture (McCollum-Davis)	Thyroid	Thio-uracil
N	12	4	73	18	5	4	—	—
HC	8	4	41	50	5	4	—	—
CF	9	4	6	50	40	4	—	—
CFa	10	10	6	50	40	4	—	—
CFT†	8	4	6	50	40	4	.45	—
CFTa†	10	8	6	50	40	4	.15	—
CFU	11	5	6	50	40	4	—	.3

* Vitamin mixture added (mg/kg) to above diet contained: riboflavin, 4; pyridoxine, 2; nicotinic acid, 25; thiamine, 50; cal. pantothenate, 20; choline chloride, 1000; biotin, .1; folic acid, 2; vit. B_{12}, .03; vit. K, 10; vit. E, 25; and cod liver oil, 2%.

† Hyperthyroid rats fed double amount of vitamin mixture.

TABLE III. Liver Glycogen, Lipids and Tissue Weights in Rats Fed High Fat-High Casein Diets.

Group	No. of rats	Feeding period in wk	Blood sugar, mg/100 ml	Liver, g/100 g wet wt of tissue		Tissue wt g/100 g body wt		Adrenals, mg/100 g body wt
				Total lipids	Glycogen	Liver	Kidneys	
N	12	4	84 ± 3	3.8 ±.3	2.45±.3	3.6 ±.2	.82 ±.01	14 ± 2
HC	8	4	94 ± 4	4.15±.4	2.3 ±.31	4.0 ±.33	.84 ±.02	16 ± 2
CF	9	4	100 ± 4	5.2 ±.4	2.4 ±.41	4.4 ±.35	.92 ±.04	19 ± 2.6
CFa	10	10	114 ± 5	6.2 ±.5	2.0 ±.32	4.7 ±.4	1.0 ±.04	20 ± 3
CFT	8	4	126 ± 6	6.8 ±.52	.5 ±.2	5.0 ±.46	1.12 ±.05	22 ± 2
CFTa	10	8	135 ± 6	7.3 ±.6	.2 ±.08	5.54±.47	1.31 ±.06	26 ± 3
CFU	11	5	100 ± 5	6.0 ±.70	2.6 ±.5	4.1 ±.4	.88 ±.05	12 ± 3

Mean values with stand. dev.

on the efficiency of food utilization in these animals.

Results summarized in Table III reveal the tendency of hyperglycemia in rats fed high casein or high fat-high casein diets. Hyperthyroidism induced by thyroid feeding (Groups CFT and CFTa) augments the existing hyperglycemia. In view of the close relationship between glycogen content and ketogenesis(7), liver glycogen estimations were made in all the rats. It is clear from the results that thyroid administration depletes liver glycogen to .5% as compared to the control value of 2.4%. Gradual onset of hyperthyroidism (Group CFTa) reduces liver glycogen further to .2%. Liver glycogen is sensitive to thyroxine which increases glycogenolysis, but in rats fed thiouracil (Group CFU) liver glycogen is slightly above normal, probably due to the slowdown of general metabolism and subsequent reduction in rate of glycogenolysis in these rats. Our observations on the influence of thyroid and thiouracil on liver glycogen are in excellent agreement with those shown by other workers(4,1).

Concentration of liver lipids in high casein and high fat-high casein fed rats is found to be increased. When these diets are continued for longer periods or fed along with thyroid, the development of fatty livers is much more pronounced. This increase in lipids is probably due to enhanced rates of hepatic fatty acid oxidation following augmented mobilization of fats to the liver from extrahepatic tissues. This effect can be brought about by a relative lack of carbohydrate in the diet of these rats in which case more fats will be oxidized for energy production and the necessary fatty acids will be mobilized from the body reserves present in adipose tissue. Weights of adipose tissues (perinephral and epididymal) in rats from Groups CFa, CFT and CFTa were found (unpublished) to be reduced by 50-60%, while in hypothyroids (Group CFU) they were nearer

TABLE IV. Oxygen Uptake, Acetoacetate Produced and Utilized by Tissue Slices.

Group	Oxygen uptake in μl of liver slices			Kidney slices, 300 μl* acetoacetate	Acetoacetate* produced by liver slices, in μl			Kidney slices acetoacetate*	
	No substrate	Acetate .08 M	Butyrate .02 M		No substrate	Acetate .08 M	Butyrate .02 M	Produced	Utilized†
N	200 ± 10	286 ± 11	344 ± 18	677 ± 19	15 ± 6	58 ± 8	79 ± 10	8 ± 5	80 ± 5
HC	204 ± 12	310 ± 12	304 ± 10	638 ± 18	26 ± 5	68 ± 7	85 ± 11	18 ± 4	94 ± 7
CF	185 ± 12	332 ± 13	397 ± 20	697 ± 21	28 ± 8	76 ± 6	100 ± 12	18 ± 5	118 ± 8
CFa	190 ± 11	370 ± 14	428 ± 21	711 ± 22	34 ± 8	100 ± 7	131 ± 15	25 ± 5	98 ± 9
CFT	280 ± 14	400 ± 16	426 ± 25	750 ± 30	60 ± 11	140 ± 20	150 ± 15	21 ± 6	127 ± 11
CFTa	310 ± 15	478 ± 20	496 ± 29	780 ± 32	115 ± 11	200 ± 21	240 ± 19	35 ± 7	102 ± 10
CFU	168 ± 9	200 ± 16	241 ± 25	580 ± 40	18 ± 5	46 ± 10	71 ± 8	42 ± 8	71 ± 12

Mean values with stand. dev. Results calculated for 100 mg wet wt of tissue per 2 hr.
Kreb's Ringer phosphate buffer pH 7.2, incubation temperature 37.5°C; oxygen as gas phase; total incubation period 2 hr.
* CO_2 in μl produced after decarboxylation of acetoacetate with aniline citrate.
† 300 μl of acetoacetate added as a substrate.

to normal values (Group N). Changes in adipose tissue weights may be a reflection of augmented fat mobilization and decreased lipogenesis *in situ*. Haist(8) observed that the lack of carbohydrate usually results in hyperlipacidemia, enhanced lipid oxidation, and ketogenesis, with an accompanying decreased rate of lipogenesis. Deuel and Hallman(9) found a correlation between liver fat and ketonuria produced in rats fed high fat diet. Chernick and Scow(10) observed that neither ketosis nor fatty livers developed in diabetic rats that were first depleted of body fat by fasting.

Increased tissue weights of liver, kidneys and adrenals in rats fed high casein-high fat diet in general indicate the dietary stress. Ohneda Akira(11) also observed hyperactivity of adrenals in rats fed such diets. Weight of adrenals in hypothyroid rats (Group CFU) is decreased but in hyperthyroid rats there is marked hypertrophy of adrenals. This difference in adrenal weights may be attributed to the sensitivity of hypothyroids and resistance of hyperthyroid rats to adrenocorticosteroid hormones as observed by Aterman(12).

Acetoacetate production and utilization. Several workers have proved that the ketone bodies are chiefly produced by liver and oxidized by extra-hepatic tissues, especially muscles and kidneys. The metabolism of ketone bodies is greatly influenced by nutritional and endocrine status of the animals. An enormous rise in endogenous and exogenous ketogenesis by liver slices of rats in Groups CF and CFT is evident from the results presented in Table IV. This over-production of ketone bodies is greater in rats fed high fat-high casein diets for 10 weeks (Group CFa) or in hyperthyroids (Group CFTa). The ketogenic effect of high casein diet observed in these experiments is less severe than that of high fat-high casein diets and may be explained as follows: Although most of the amino acids present in a protein (casein) are glycogenic, leucine, phenylalanine, tyrosine and norleucine are converted to acetoacetate in the course of their metabolism in an intact animal or in an isolated tissue(13). Edson (14) has shown that ammonia is ketogenic

and that the ammonia produced by the deamination of amino acids acts similarly to exogenous ammonia. Hence, when amino acids form a larger proportion of the substrates of the liver cell, the increased amount of ammonia liberated may play a role in stimulating ketogenesis. According to Recknagel and Potter(15), the mechanism of this effect depends upon formation of glutamic acid from the ammonia and alpha-ketoglutaric acid. This reduces the amount of oxalacetate reformed in the TCA cycle and, therefore, 'active acetate' is diverted from oxidation to acetoacetate synthesis. Thyroxine is known to increase protein catabolism and deamination of amino acids and, therefore, the livers of hyperthyroid rats (Groups CFT and CFTa) produce more ketones than their respective controls (Group CF and CFa). Bratzler *et al.*(16) have reported inhibitory effects of hyperthyroidism on lipogenesis. There is ample evidence to show that the feeding of high fat diets to animals increases hepatic fatty acid oxidation(17) and ketogenesis(18) accompanied by a decrease in lipogenesis(19) and glucose utilization(20). Insulin content(8) and the number of B-cells(21) in the pancreas of such rats were also found to be low.

Thus over-production of acetoacetate may be a reflection of enhanced rate of hepatic fatty acid oxidation coupled with reduced lipogenesis in the rats fed high fat-high casein diets alone or with thyroid. This explanation finds support in our experiments carried out to study lipogenesis in adipose tissue of rats fed different diets(22). It is generally recognized that rate of substrate utilization is ordinarily directly dependent upon substrate concentration, within certain physiological limits and, therefore, the rate of ketone body utilization is a function of their level in blood. The results presented here (Table IV) and those reported previously (1), show that over-production of acetoacetate by liver results in its increased utilization by kidney slices of rats fed a high fat-high casein diet alone or with thyroid. However, the rate of utilization is not maintained at that high level on prolonged feeding of the same diets. This effect is probably due to continued over-strain and subsequent damage to tissue caused by the higher concentration of ketone bodies. Schwab and Lotspeich(23) indicate that acetoacetate is a threshold substance causing damage to kidney tubules followed by ketonuria. Our analysis revealed 3-4 times more ketone bodies and appreciable quantities of sugar in the urine of rats in Groups CFa and CFTa as compared to their respective controls in Groups CF and CFT. Continued overproduction of acetone bodies in rats fed different diets and in hyperthyroidism, is apt to disturb the normal homeostasis in these animals. Various deleterious effects of ketone bodies on carbohydrate metabolism have been reported by Nath *et al.*(24-27) and by others(11,28).

Summary. The results in general indicate that the antecedent diet leaves its imprint on the metabolism of isolated tissues, causing profound changes in the metabolic pattern, depending on the type of such diet. The gain in body weight of rats fed high casein and high fat-high casein alone or in combination with thyroid, is found to be less than that in normals. These rats were observed to have in varying degrees hyperglycemia, fatty liver and hypertrophy of liver, kidney and adrenal tissue. Feeding of high fat-high casein diet alone, or with thyroid, induces overproduction of acetoacetate, both endogenous and exogenous, by liver slices. Utilization of acetoacetate by the kidney slices obtained from such rats is not, however, increased. Consumption of oxygen by liver and kidney slices of rats fed high casein and high fat-high casein is less than that in normal tissues. The metabolic disturbances observed in the tissues of rats fed diets low in carbohydrate but rich in protein or fat plus protein, are aggravated by prolonged feeding of the same diet (10 weeks as compared to 4 weeks).

1. Khanade, J. M., Nath, M. C., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 566.

2. Nath, M. C., Khanade, J. M., *ibid.*, 1960, v103, 559.

3. Deuel, H. J., Jr., Gulick, M., Butts, J. S., *J. Biol. Chem.*, 1932, v98, 333.

4. Houssay, B. A., *Vitamins and Hormones,* 1946, vIV, 196.

5. McCullum, Davis, *J. Biol. Chem.,* 1918, v33, 55.
6. Bosshardt, D. K., Ayres, M. M., Ydse, L. C., Barnes, R. H., *J. Nutrition,* 1946, v32, 93.
7. Mayes, P. A., *Nature,* 1958, v182, 324.
8. Haist, R. E., *Physiol. Rev.,* 1944, v24, 409.
9. Deuel, H. J., Jr., Hallman, L. F., *J. Biol. Chem.,* 1941, v140, 545.
10. Chernick, S. S., Scow, R. O., *Am. J. Physiol.,* 1959, v196, 125.
11. Ohneda Akira, *Tohoku J. Exp. Med.,* 1960, v72, 107.
12. Aterman, K., *Endocrinology,* 1957, v60, 711.
13. Lerner, A. B., *Advances in Enzymology,* 1953, v14, 73.
14. Edson, N. L., *Biochem. J.,* 1935, v29, 2082; 2498.
15. Recknagel, R. O., Potter, V. R., *J. Biol. Chem.,* 1951, v191, 263.
16. Bratzler, J. W., Barnes, J. R., Swift, R. W., *J. Nutrition,* 1949, v39, 41.
17. Tepperman, J., Tepperman, H. M., Schulman, M. P., *Am. J. Physiol.,* 1958, v184, 80.
18. Roberts, S., Samuels, L. T., *J. Biol. Chem.,* 1943, v151, 267.
19. Masoro, E. J., Chaikoff, I. L., Chernick, S. S., Felts, J. M., *ibid.,* 1950, v185, 845.
20. Whitney, J. E., Roberts, S., Beaver, E. L., *Am. J. Physiol.,* 1955, v182, 51.
21. Vartiainen Ilmari, Talkka Antti, *Ann. med. int. Fenniae,* 1955, v44, 79.
22. Khanade, J. M., Nath, M. C., *Am. J. Physiol.,* in press.
23. Schwab, Louis, Lotspeich, W. D., *ibid.,* 1954, v176, 195.
24. Nayudu, S. G., Nath, M. C., *J. Sci. Ind. Res.,* 1958, v17C, 172.
25. Nath, M. C., Chakrabarti, C. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 326.
26. Nath, M. C., Sahu, V. K., Chitale, R. P., *Biochem. J.,* 1953, v53, 684.
27. Nath, M. C., Hatwalne, V. G., Chitale, R. P., *ibid.,* 1953, v53, 481.
28. Schwastz, *Nature,* 1960, v185, 772.

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Alterations in Growth of Strain L Cells Exposed to Smoke Gases.* (27405)

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The purpose of this investigation was to observe the effect of smoke and certain non-smoke gases on growth of cells in culture. Our interest in this subject was stimulated by the over-all problem of cancer of the lung and by the fact that only limited studies on the effects of smoke on single cells have been published. Cigarette smoke bubbled through a culture of certain pathogenic bacteria in a test tube killed the bacteria(1). The interfering action of cigarette smoke on the ciliated mucous secreting respiratory epithelium *in vitro* has also been established(2). The particulate phase of cigarette smoke over and above the chemistry of smoke has been implicated in the interference of normal functioning of respiratory epithelium(3).

The present series of studies was undertaken further to elucidate the direct action of smoke on living cells.

Materials and methods. Strain L cells designated 929-421-303, obtained from Dr. W. R. Earle, were maintained in 2 cc of medium NCTC-109, with 10% horse serum in B-15 Bellco Flasks with the pH adjusted to 7.4. The flasks were seeded with 50,000 cells/ml. The doses of smoke used were 0.5 cc, 1.0 cc, and 1.5 cc at N.T.P. After preparation the flasks were allowed to remain stationary in the incubator at 37.5°C. At the end of 24 hours the cells had adhered to the glass, and were well spread out, but had not completed their first division cycle. Smoke was collected under sterile conditions in a syringe from a modified Phipps and Bird smoking apparatus. A sterile side arm was installed and the smoke was collected directly at its source. The flasks were inverted and the prescribed

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