Persistence of Lipolysis in Frozen Adipose Tissue[1] (34483)

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In studying the regulation of lipolysis in adipose tissue, it is often necessary to postpone the chemical analyses of tissue and incubation medium for some time after the termination of the experiment. For this purpose, we have stored such tissues and media in a freezer at −8°. It was soon observed, however, that experiments in which samples were frozen for more than a day or two prior to analysis gave particularly high values for both free fatty acids (FFA) and glycerol. For the past few years, therefore, we have routinely boiled the adipose tissue segments prior to freezing, and have thereby obviated the problem. The present report documents these findings.

Materials and Methods. Normal adult male rats (180–240 g) of the Holtzman strain were used in these experiments. Animals designated as fed were allowed free access to Purina Lab Chow and water up until the time of sacrifice. "Fasted" animals were deprived of food 16–18 hr prior to sacrifice. The rats were killed by cervical fracture, and the epididymal fat rapidly excised and dissected into 2–4 segments weighing approximately 50 mg each. Only the thin, distal portions of the epididymal fat were used. In the first experiment, two tissue segments from each rat were placed separately in 1 ml of distilled water in a 12-ml centrifuge tube. One tissue from each rat was immediately placed in a boiling water bath for 3 min. Both tissues were then frozen without incubation. Two additional tissues from each rat were placed in incubation vials which contained 1 ml of Krebs-Ringer bicarbonate buffer and 40 mg of bovine serum albumin (Armour, Fraction V). The vials were stoppered, gassed with 95% O_2:5%CO_2, and incubated for 1 hr at 37°. At the end of the incubation period, the tissues were removed from the vials and otherwise handled as above. In a second experiment, three tissues from each rat were incubated for 1 hr in Krebs-Ringer bicarbonate buffer containing 4% bovine serum albumin and 1 mg/ml of glucose, conditions which have become standard for this laboratory. At the end of the incubation, one vial representing each rat was placed, unopened, in the freezer. The second incubation vial prepared from each rat was opened, and the tissue segment removed for immediate analysis of its content of FFA. The vials containing just the incubation medium were resealed and frozen for 10 days. The third tissue segment from each rat was transferred from the incubation medium to 1 ml of distilled water, placed in a boiling water bath for 3 min, and then stored in the freezer for 10 days. The incubation media from these tissues were discarded. All the tissues were homogenized in 5 ml of Dole's extraction mixture (1) and their content of FFA analyzed according to the Dole procedure (1). Aliquots of incubation medium were also analyzed for their content of FFA by the Dole procedure and for glycerol by the method of Wieland (2).

Results. After storage at −8° for 10 days, the FFA content of boiled tissues obtained from either fed or fasting rats was lower than that of corresponding tissues which had not been boiled for 3 min prior to storage (Table I). The boiled tissues gave values that agreed very well with data routinely obtained in this laboratory with adipose tissue treated

[1] Supported by Grant Am 06935 from the National Institutes of Health.

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[3] Recipient of Career Development Award K3 7797 from the National Institutes of Health.

TABLE I. The Effects of Prior Boiling on FFA Concentration of Adipose Tissue Segments Stored at —8° for 10 Days.

	FFA concentration (μeq/g)[a]		
	Boiled	Not boiled	p
Fed rats			
Unincubated	1.01 ± 0.24	4.37 ± 0.27	<.001
Incubated	1.01 ± 0.16	1.31 ± 0.13	NS[b]
Fasting rats			
Unincubated	3.40 ± 0.25	8.39 ± 0.60	<.001
Incubated	2.45 ± 0.29	3.81 ± 0.23	<.01

[a] Mean ± SEM, 8 observations.
[b] Not statistically significant ($p > .05$).

similarly, but analyzed immediately. (Table II). The differences seen were considerably greater in tissues frozen immediately after excision than in tissues which had been incubated *in vitro* for 1 hr. In unincubated tissues of fed rats, the boiled tissues had only one fourth of the FFA content of the unboiled tissues. The small difference (0.30 μeq/g) between boiled and unboiled tissues which had been incubated was not statistically significant in this experiment. The difference between the FFA content of boiled and unboiled tissues of fasting rats was more than three times greater in unincubated than in incubated tissues (5.01 μeq/g vs 1.36 μeq/g).

To determine whether the differences between boiled and unboiled tissues actually reflects lipolysis and not some other, nonspecific process (e.g., oxidative cleavage of unsaturated fatty acids), we measured the production of glycerol as well as FFA after storage for 10 days in the freezer (Table II). Tissues stored unboiled in the freezer contained significantly more FFA than replicate tissues analyzed immediately after incubation or boiled and analyzed after 10 days' storage in the freezer. Allowing the unboiled tissues to remain in the incubation medium during the 10 days of storage in the freezer resulted in highly significant increases in the release of glycerol into the incubation medium, but had no effect on the release of FFA.

Discussion. The lipolytic process persists, albeit at a reduced rate even in tissues stored at —8°. It is therefore not possible to obtain reliable data on glycerol and FFA production if tissues are stored frozen for any length of time before chemical analyses are made. The errors introduced will be greater in unincubated than in incubated tissues, and, on a percentage basis at least, greater in tissues obtained from fed than from fasting rats. These errors can be eliminated, however, by briefly heating the tissues in a boiling water bath prior to storage in the freezer.

TABLE II. The Effects of Storage for 10 Days at —8° on Various Indices of Lipolysis.

	Tissues removed immediately after incubation	Tissues frozen in incubation medium	p
Fed rats			
Tissue FFA (μeq/g)	0.63 ± 0.07[a,b] 0.63 ± 0.09[c]	1.24 ± 0.15	<.01
FFA released (μeq/g)	0.80 ± 0.24	0.49 ± 0.17	NS[d]
Glycerol released (μmoles/g)	1.19 ± 0.21	2.61 ± 0.28	<.001
Fasting rats			
Tissue FFA	1.67 ± 0.25[b] 1.38 ± 0.16[c]	3.44 ± 0.24	<.001
FFA released	1.61 ± 0.36	1.68 ± 0.17	NS[d]
Glycerol released	2.63 ± 0.20	3.98 ± 0.16	<.001

[a] Mean ± SEM, 8 observations.
[b] Homogenized and analyzed immediately.
[c] Boiled, frozen, and analyzed 10 days later; the FFA content of these tissues did not differ significantly from those analyzed immediately.
[d] Not statistically significant ($p > .05$).

If it may be assumed that 3 moles of FFA are formed for every mole of glycerol released, the data presented in Table II suggest that reesterification of FFA as well as lipolysis may continue even in frozen tissues. In the tissues of fed rats, 1.42 moles/g of glycerol were formed in the freezer. If no reesterification occurred, and if only the complete hydrolysis of triglycerides led to glycerol and FFA release, the FFA content of the tissue and medium should have increased by 4.26 μmoles. The actual increase in FFA, however, was only 0.58 μmoles. Similarly, in tissues of fasting animals, 1.35 μmoles of glycerol were formed in the period in which the tissues were frozen, while the increase in FFA in the tissue and medium was only 1.84 μmoles instead of the theoretical 4.05 μmoles.

The explanation for higher rates of FFA formation by unincubated tissues is unknown. However, it is known that lipolysis decreases sharply with time of incubation [Geyer and Goodman, unpublished, and (3)]. Whether such a decrease is due to the gradual destruction of some endogenous lipolytic stimulus, to an inactivation of the lipase itself, or to some combination of these two is unknown. In any event, it is quite possible that the processes leading to the reduced activity of the lipase *in vitro* may be more susceptible to cold than the lipolytic process itself. If this is true, "incubation" of tissues at low temperatures might provide a useful means for studying differences in initial rates of lipolysis in tissues removed from rats in different physiological states.

Another possible application of these findings arises from the observation (Table II) that while the FFA content of the tissues increased by at least 2-fold while the tissues were frozen, the FFA content of the medium bathing the tissues remained unchanged. Although glycerol diffused out of the tissues into the medium, the newly formed FFA remained within the tissues. While these findings may be artifactual, it is entirely possible that the egress of FFA from adipose tissue is an active process which is more susceptible to low temperatures than is lipolysis. If this proves to be the case, "incubation" of adipose tissue at low temperature might provide a tool for studying the active outward transport of FFA.

Summary. Segments of epididymal fat continue to produce FFA and glycerol during storage in the freezer at —8°. This effect is seen in tissues of both fed and fasted rats, and is most pronounced in tissues which have not been incubated prior to freezing. Lipolysis was prevented by placing the tissues in a boiling water bath for 3 min prior to storage in the freezer.

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Received Sept. 8, 1969. P.S.E.B.M., 1970, Vol. 133.