Studies on Volatile Fatty Acid Oxidation and Incorporation into the Lipid of Liver Slices from Alloxan Diabetic and Starved Sheep

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Studies on Volatile Fatty Acid Oxidation and Incorporation into the Lipid of Liver Slices from Alloxan Diabetic and Starved Sheep

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Summary

1. The liver slices from alloxan diabetic and starved ewes were incubated with C^{14} labeled volatile fatty acids, glucose and glutamic acid to investigate both the incorporation of substrates into the lipid and the oxidation to C^{14}-carbon dioxide.

2. Acetate and butyrate had less consumption in livers from alloxan diabetic and starved animals than in the control animals, whereas propionate consumption some what increased under both conditions.

3. The productions of $C^{14}O_2$ from all of the substrates in the liver slices from alloxan diabetic and starved animals decreased significantly as compared with that in normal liver slices. The oxidation rate of volatile fatty acids was apparently higher than that of glucose and glutamic acid in all liver slices examined.

4. In normal liver slices, the incorporation of each substrate into the lipid was only a small per cent of the substrate added to the medium.

5. Although the lipid contents of liver slices markedly increased by alloxan diabetes and starvation, the incorporation of C^{14} substrate into the lipid of those liver slices was significantly lower than in normal liver.

The volatile fatty acids produced by microbial fermentation of the food in the rumen are the principal source of energy for the ruminant (1). It is well recognized that of the volatile fatty acids produced in and absorbed from the rumen, acetic and butyric acids or approximately 80 per cent are ketogenic and only propionic acid is glycogenic (2, 3, 4, 5). Therefore the nature of the rumen fermentation, that is the quantities and the proportions of volatile fatty acids, may lead to the alteration of the nutritional state of the ruminant.

On the other hand, the ruminant is forced to produce large amounts of the milks and to maintain the foetus during pregnancy. These producing performances should be regarded as their physiological sacrifice.

Because of these facts, in high-producing cow or multiparous ewes various nutritional disturbances frequently occur. Although many approaches have been

made to understand the biochemical and physiological mechanisms involved in the development of nutritional disorders, many questions still remain to be sloved.

Hitherto a large part of the studies on volatile fatty acid metabolism have been concerned with ketone body formation and glucose synthesis, and less attention has been given to lipogenesis in the liver.

The purpose of our study is to know the metabolic patterns of volatile fatty acids during changing hormonal and the nutritional conditions. The present report is concerned with the effects of alloxan diabete and starvation on the oxidation of lebeled volatile fatty acids (VFAs) and the incorporation into the lipid in liver slices of sheep.

Materials and Methods

Animals and treatments — Seven Merino ewes weighing between 40 and 50 kg were used for the experiment. They were fed 600 g of hay and 400 g of concentrates daily. Three of them were injected intravenously with alloxan (75 mg/kg body weight) dissolved in 50 ml of saline and were supplied 4 days after the injection for the experiment. Two other ewes of them were starved for twenty days with only water supplied *ad libitum*. Two another ewes were used as contral animals. All of these animals were killed by exanguination via the carotid artery for studies in vitro.

Incubation procedures — The liver was removed immediately after death and immersed in cold 1.15% KCl solution. The liver slices were incubated in 50 ml Erlenmeyer flasks for lipid and VFA analysis and in 25 ml Erlenmeyer flasks with center well for the CO_2 assay with 1-C^{14} labeled acetic, propionic and butyric acids, or U-C^{14} labeled glucose and glutamate (50 μmoles of each substrate contain 0.1 μc of radioactivities). These vessels contained 50 μmoles of substrate and 3 ml of Krebs Ringer bicarbonate buffer solution, pH 7.4, and 0.5 g wet weight of liver slices.

The flasks were flushed with O_2+CO_2 (95:5) for 5 min, capped with tightly fitting rubber serum stoppers and incubated for 3 hr. at 37°C.

CO_2 assay — After the incubation, 0.5 ml of Hyamine was injected through the cap into the center well to collect CO_2 and 0.5 ml of 5N H_2SO_4 was injected into the medium to stop the reaction. Flasks were then shaken for 60 min. at below 5°C before removal of the Hyamine to a scintillator in a vial for counting.

VFAs assay — The incubation mixture was centrifuged at 3000 r.p.m. for 15 min. An aliquote of the supernatant obtained by the centrifugation was used for the VFAs analysis. The VFAs were determined by silicic acid chromatography by the method of Belasco (7). The VFAs consumption during the incubation were calculated by subtracting the amounts of VFAs remaining in the medium from the VFA added before the incubation.

Lipid assay — Lipid was extracted from the tissue by the method of Folch (8). An aliquote of extract was dried under nitrogen gas airation and was weighed. For the measurements of the incorporation of substrates into the lipid, the dried lipid samples were dissolved in 2 ml of the solvent containing 2 parts of chloroform and 1 part of methanol. Then the radioactivity was measured by the liquid scintillation counting procedure. The other aliquotes of lipid extracted were separated into several components of lipid by using silicic acid chromatography by the method of Ichida (9).

Counting procedure — The radioactivity was counted with a Nuclear Chicago Liquid-Scintillation counter (Model 724) in scintillation solution containing 100 mg of POPOP and 5 g of PPO in 1 liter of toluen.

Results and Discussion

A: *Effects of alloxan injection and starvation on various constituents of the blood and the rumen contents.*

1. *Effects of alloxan injection*

With alloxan injection, the blood sugar concentration reached 200 mg/dl within 6 hrs after the injection and maintained this high level for 96 hrs. Non-esterified fatty acid (NEFA) in the blood increased remarkably at 24 hrs after the

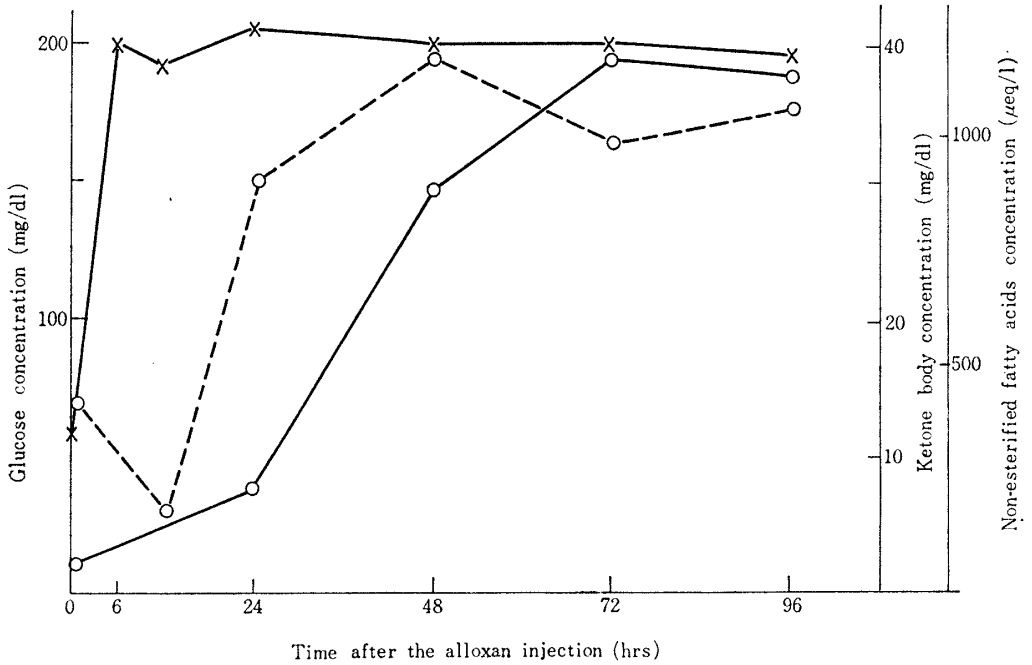

Fig. 1. Effects of alloxan injection on blood constituents of sheep. Animal was injected intravenously with alloxan (75 mg per kg of body weight), which dissolved in 50 ml saline.

× —— × ; glucose. ○ —— ○ ; ketone body. ○ - - - - ○ ; non-esterified fatty acids.

injection, then reached about 1000 μeq/l. Blood ketone body concentration gradually increased and 5 days after reached 30 mg/dl as shown in Fig. 1.

It is considered from the obatained results that since alloxan treatment results in the inhibition of insulin secretion from the pancreas, glucose cannot be utilized sufficiently by the tissues and as a result the glucose was accumulated in the circulating blood. The extreme increase of NEFA in the blood suggests that there occured an increased mobilization of the extrahepatic fat stores to the liver. Krebs who studied using the diabetic rats suggested that since the tissues of the diabetic animals has a remarkable requirment for glucose, oxaloacetate may be used toward gluconeogenesis rather than condensation with acetyl-CoA and that under such a circumstance the acetyl-CoA may be forced to condese itself and thus formed ketone bodies. Our results presented above could be understood by his explanation.

2. *Effects of starvation*

As shown in Fig. 2, the blood NEFA concentration increased from 200 μeq/l initialy to 1000 μeq/l in 5 days after fasting and this high level was maintained during the 20 days of fasting. The volatile fatty acid concentration in the rumen juice decreased to below 100 mg/dl by 10 days after fasting, indicating there is no doubt that animals are not supplied the nutrients from rumen for maintenance.

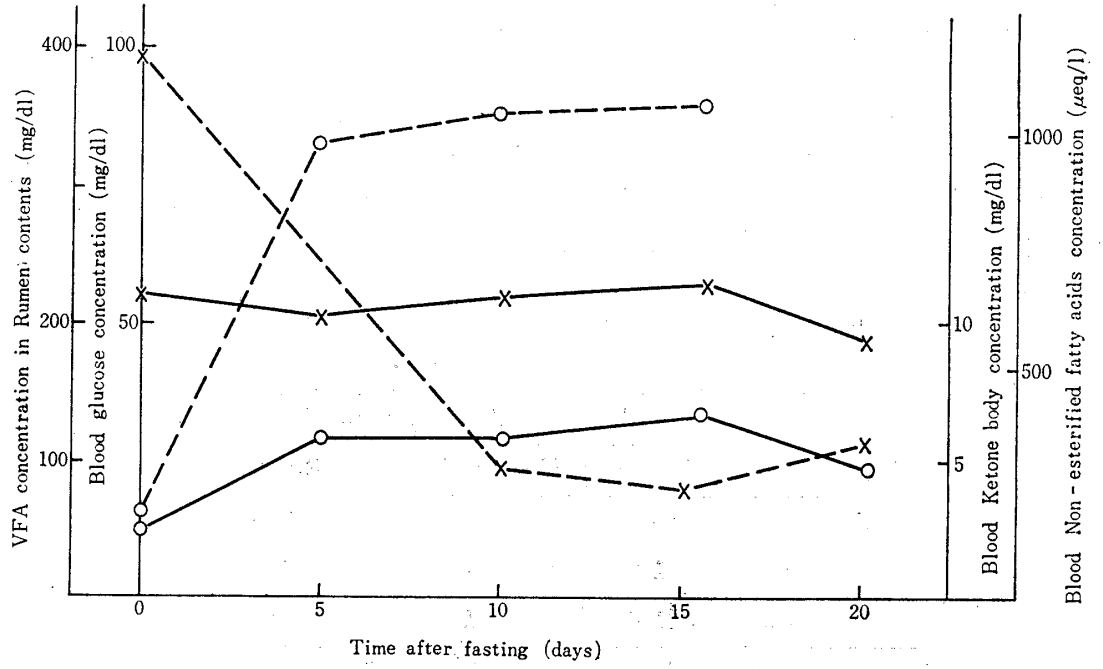

FIG. 2. Effects of fasting on blood constituents and rumen volatile fatty acids of sheep.
×——×; blood glucose. o——o; blood ketone body. o- - -o; blood non-esterified fatty acids. ×- - - -×; volatile fatty acid in rumen contents.

Whereas the concentrations of blood glucose and blood ketone bodies were almost unchanged during the fasting.

These facts suggest that glucose formation from certain substances other than VFAs is initiated during the fasting. Shiga, who studied the employment of the arterio-veneous difference method, found that in starved goats blood sugar concentrations were not decreased even though the losses of body weight attained 30%, and that amino acids were released from their hind leg tissues into the veneous blood of the leg.

It is possible that under such conditions, the enhenced oxalacetate formation occurs from various substances such as glucogenic amino acids which are released from the extrahepatic tissues mainly the muscle and that such metabolic changes could lead to an increase glucose formation rather than ketone body formation. The observed increases in blood NEFA concentration may be due to depot fat mobilization under instructions which are induced by the limited glucose degradation.

B: *The effects of alloxan administration and starvation on volatile fatty acids metabolism in the liver slices of sheep.*

1. *VFA consumption in the liver slices.*

VFA consumption of liver slices from sheep under various physiological conditions were given in Table 1.

The consumption of acetate and butyrate in the liver slices reduced significantly with alloxan administration. Similar results were obtained in the liver slices of 20 day-fasted ewes.

It should be noted carefully that the oxidation of labeled substrates to CO_2 decreased in the liver slices with treatments, since the radioactivity from substrates decreased because of the increased dilution in a larger acetyl-CoA pool during mobilization of NEFA from the depot fat.

TABLE 1. *Consumption of Volatile Fatty Acids in Sheep Liver Slices.*

Sheep / Substrates	Substrates Consumption (%)		
	Normal	Alloxan diabteic	Starved
Acetate	62.0	34.0	38.0
Propionate	71.0	82.0	75.4
Butyrate	61.6	41.0	44.0

Five hundred mg of liver slices were incubated with 50 μmoles of each substrate in 3 ml of Krebs Ringer bicarbonate buffer at 37°C for 3 hr.
The figures represent the percentage of consumption to added substrates.

However propionate consumption did not change or even increase. Acetate and butyrate enter the metabolic pathway as TCA cycle via acetyl-CoA, if acetyl-CoA formation from NEFA increases, the acetyl-CoA formation from acetate and butyrate will be somewhat blocked. These our results might be influenced by this action. While propionate consumption might be increased by gluconeogenic stimulation.

2. *CO_2 production from various substrates in liver slices.*

Table 2 shows the CO_2 productions from various substrates with the liver slices of alloxan diabetic and starved ewes. The CO_2 production from all of the substrates decreased significantly more than with normal liver slices. The CO_2 production from glucose and glutamic acid was less than derived from VFAs. Bruggen found that in the liver slices from alloxan diabetic rats total CO_2 production decreased, whereas incorporation of radioactivities into CO_2 increased. There-

TABLE 2. *CO_2 Productions from Various Substrates in Sheep Liver Slices.*

Sheep / Substrates	$C^{14}O_2$ production (μmc)		
	Normal	Alloxan diabetic	Starved
Acetate	22.3	4.6	2.2
Propionate	57.7	43.0	25.0
Butyrate	14.4	11.2	8.0
Glucose	1.6	0.9	0.8
Glutamate	1.6	4.9	1.3

Five hundred mg of liver slices were incubated with 50 μmoles of each substrate containing 0.1 μc of C^{14} in 3 ml of Krebs Ringer bicarbonate buffer at 37°C for 3 hr.

fore it may be questionable to judge that the oxidation to CO_2 of these substrates were decreased only from our results, because the radioactivities from labeled substrates might be diluted owing to fatty acid mobilization from depot fat because of the acetyl-CoA pool increase. Accordingly it might be interpreted that the $C^{14}O_2$ production decreased. While the $C^{14}O_2$ production from propionate was larger than that from other VFAs, it may be thought that the radioactivities of propionate were not diluted since propionate is not oxidized via acetyl-CoA. However these results may depend on decreased metabolic activities in liver slices, as Pritchard pointed out (17). The similar results were reported by Leng and Annison (18), but they reported that $C^{14}O_2$ production from propionate decreased on fasted sheep liver slices. Their finding are discordant with the result in the present work.

3. *Lipid contents and its composition in liver slices*

The lipid content in the liver slices are shown in Table 3. Five hundred mg of the normal liver slices contained 19 mg, the alloxan diabetic 38 mg, and the starved 50 mg. These later two states showed remarkably higher levels of liver lipid.

The composition of liver lipid is shown in Fig 3. Normal liver contained 63% phospholipid, 20% cholesterol, 12% triglyceride, while the alloxan diabetic liver contained 36% phospholipid, 8% cholesterol and 52% triglyceride. In the alloxan diabetic condition, the liver lipid contained much triglyceride but the phospholipid did not quantitatively increased. This increased triglyceride would be due to the transportation of NEFA from depot fat via the blood stream. These findings are in accord with expectations base upon known sequences of intermediary metabolism.

TABLE 3. *Lipid Contents of Liver Slices from Various Sheep.*

Sheep / Substrates	Lipid contents (mg)		
	Normal	Alloxan diabetic	Starved
Acetate	20.1	38.9	56.2
Propionate	19.1	39.9	49.6
Butyrate	18.6	39.8	46.5
Glucose	19.0	35.8	41.5
Glutamate	16.7	36.1	53.3

Five hundred mg of liver slices were incubated with 50 μmoles of each substrate in 3 ml of Krebs Ringer bicarbonate buffer at 37°C for 3 hr.
The figures represent mg of lipid per 500 mg of wet tissue.

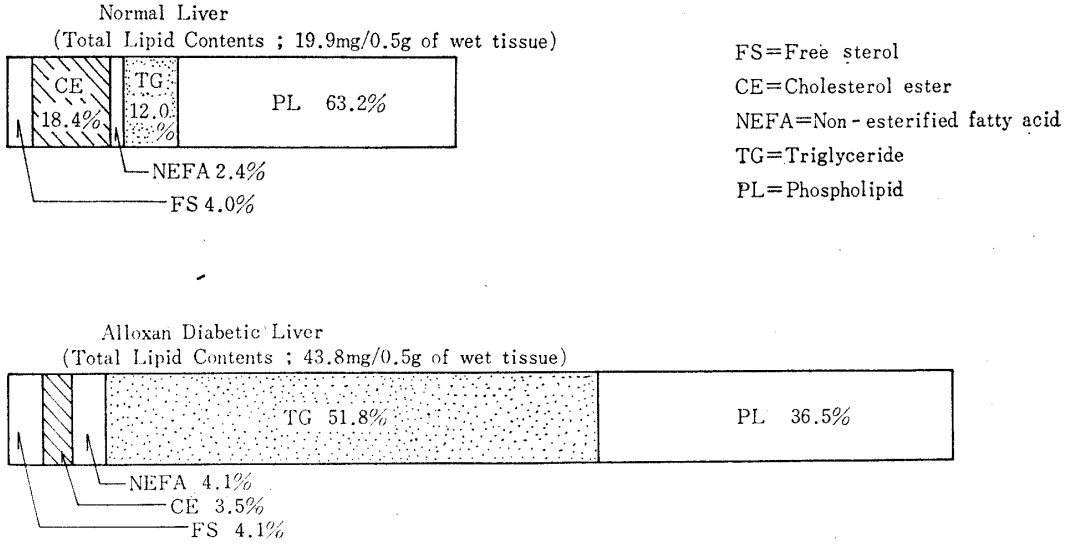

FIG. 3. Lipid composition of livers from alloxan diabetic and normal sheep.

4. *Incorporation into the liver lipid from various substrates*

The incorporation of C^{14} from various substrates into lipid in the liver slices are shown in Table 4. In the normal liver slices the incorporation from acetate was larger than from propionate and butyrate, and the incorporations from glucose and glutamic acid were significantly less than that from VFA substrates. In

TABLE 4. *Incorporation of Various Substrates into Lipid in Sheep Liver Slices.*

Sheep / Substrates	Incorporation into lipid (μmc)		
	Normal	Alloxan diabetic	starved
Acetate	3.1	0·1	0·1
Propionate	1.3	0·2	0.2
Butyrate	1.2	0·1	0.2
Glucose	0.2	0·1	0·1
Glutamate	0.2	0·1	0·4

Five hundred mg of liver slices were incubated with 50 μmoles of each substrate containing 0.1 μc of C^{14} in 3 ml of Krebs Ringer bicarbonate buffer at 37°C for 3 hr.

alloxan diabetic and starved liver slices the incorporation from all substrates were markedly decreased below normal. A partial explanation for this finding may reside in the fact that liver tissues of alloxan diabetic and starved animals had greater amounts of lipid in itself than normal tissue. However, even if these figures were corrected to the figures based on fat free tissue weight, still the incorporation into lipid from these substrates decreased in alloxan diabetic and starved liver. Many workers have reported that in the alloxan diabetic rat (13) (14) (15), the lipid synthesis decreased in the liver in vitro or in vivo. In alloxan diabetic liver the carbohydrate metabolism is evidently disturbed, especially the glycolytic pathway. The inhibition of the pentose phosphate pathway will cause a deficient supply of NADPH, which is required for the formation of fatty acids or cholesterol. The similar lack of NADPH might be induced in starved liver. A decreased incorporation of C^{14} from all substrates into lipid observed in the present work are in accord with such expectation. However, anyhow even in normal liver slices the incorporation into lipid was only a small percent of the added substrates. It would seen that the liver may not be important in the lipid synthesis from VFAs in sheep.

5. *Incorporation into each lipid fraction from various substrates*

The incoporation of C^{14} into each lipid fraction from various substrates are

TABLE 5. *Incorporation of VFAs into Each Lipid Fraction in Sheep Liver Slices.*

Added substrate	Sheep	Lipid Fraction				
		Cholesterol ester	Tri glyceride	Free cholesterol	Free fatty acid	Phospho lipid
Acetate	Normal	23.5	10.4	24.1	5.9	36.1
	Alloxan diabetic	20.2	1.7	0	52.0	26.0
	Starved	22.0	13.8	10.6	20.2	45.9
Propionate	Normal	18.5	9.7	17.3	10.7	43.7
	Alloxan diabetic	9.2	26.7	12.3	11.5	41.3
	Starved	12.7	0	6.4	19.5	61.4
Butyrate	Normal	8.4	9.1	7.1	50.8	24.6
	Alloxan diabetic	5.1	1.5	24.5	17.9	51.0
	Starved	56.9	0	3.6	13.9	25.5

Five hundred mg of liver slices were incubated with 50 μmoles of each substrate containing 0.1 μc of C^{14} in 3 ml of Krebs Ringer bicarbonate buffer at 37° C for 3 hr.
The figures represent the percentage of incorporated substrates into each fraction to total lipid.

TABLE 6. *Incorporation of Glucose and Glutamate into Each Lipid Fraction in Sheep Liver Slices.*

Added substrate	Sheep	Lipid Fraction				
		Cholesterol ester	Tri glyceride	Free cholesterol	Free fatty acid	Phospho lipid
Glucose	Normal	11.1	22.3	11.9	8.3	46.9
	Alloxan diabetic	6.8	27.7	7.5	17.7	40.3
	Starved	66.7	0	0	0	33.3
Glutamate	Normal	8.5	9.3	15.0	22.0	45.2
	Alloxan diabetic	4.3	16.3	6.4	23.8	49.4
	Starved	5.4	7.7	2.3	19.3	65.3

Five hundred mg of liver slices were incubated with 50 μmoles of each substrate containing 0.1 μc of C^{14} in 3 ml of Krebs Ringer bicarbonate buffer at 37°C for 3 hr.
The figures represent the percentage of incorporated substrates into each fraction to total lipid.

shown in Table 5 and 6. Acetate was incorporated into mainly cholesterol and phospholipid in the normal liver slices, into NEFA and phospholipid in alloxan

diabetic, and into cholesterol and phospholipid in starved. Propionate was incorporated into cholesterol and phospholipid in the normal liver slices, into triglyceride and phospholipid in alloxan diabetic, and into NEFA and phopholipid in starved, respectively. Butyrate was incorporated considerably into NEFA and phospholipid in normal liver slices. In alloxan diabetic and starved liver slices it was incorporated into cholesterol and phospholipid. Glucose was incorporated into triglyceride and phospholipid in normal and alloxan diabetic liver slices. Glutamic acid had no differences in the incorporation patterns into lipid fraction between normal, alloxan diabetic and starved. It was incorporated into NEFA and phospholipid.

Though these results are insufficient to prove the incorporation patterns of the various substrates, yet the C^{14} from the labeled substrates were found largely in phospholipid fraction under the various conditions. Even when the lipid synthesis decreased the incorporation into phospholipid continued. This may suggests that phospholipid plays an important role in lipid metabolism of sheep.

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References

1) Stewart, W.E., Stewart, D.G., Schultz, L.H., *J. Anim. Sci.*, **17**, 723 (1958)
2) Black, A.L., *J. Biol. Chem.*, **236**, 2399 (1961)
3) Bergmann, E.N., *Amer. J. Physiol.*, **221** (3) 793 (1966)
4) Pennington, R.J., *Biochem, J.*, **58**, proc. vii (1954)
5) Seto, K., Tsuda, T., and Umezu, M., *Tohoku J. Agric. Res.*, **6**, 91 (1955)
6) Shinozaki, K., *Jap. J. Zootech. Sci.*, **37**, (8), 273 (1966)
7) Belasco, I.J., *J. Anim. Sci.*, **13**, 748 (1954)
8) Folch, J., LEES, M., *J. Biol. Chem.*, **226**, 497 (1957)
9) Ichida, T., *Hokkaido J. Med. Soc.*, **38**, 55 (1963)
10) Krebs, H.A., *Proc. Roy. Soc.*, Series B., **159**, 545 (1964)
11) Elwood, J.C., Van Bruggen, J.T., *J. Biol. Chem.*, **235**, (3), 568 (1960)
12) Elwood, J.C., Alicia Marco, and Van Bruggen, J.T., *J. Biol. Chem.*, **235**, (3), 573 (1960)
13) Shaw, W.N., Dituri, F., and Gurin, S., *J. Biol. Chem.*, **226**, 417 (1957)
14) Van Bruggen, J.T., Yamada, P., and Hutchens, T.T., *J. Biol. Chem.*, **209**, 635 (1954)
15) Baker, N., Chaikoff, I.L., and Shusdeck, A., *J. Biol. Chem.*, **194**, 435 (1952)
16) Shiga, A., Ohtomo, Y., Ambo, K., and Tsuda, T., *Tohoku Univ. Lab. Anim. Physiol. Kenkyu-Shuroku* **2**, 25 (1967)
17) Pritchard, G.I., Tove, S.B., *Biochem. et Biophy. Acta*, **41**, 130 (1960)
18) Leng, R.A., Annison, E.F., *Biochem. J.*, **86**, 319 (1963)