Reversible Inhibition of Adenine Nucleotide Translocation by Long Chain Fatty Acyl Coenzyme A Esters in Liver Mitochondria of Diabetic and Hibernating Animals*

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SUMMARY

The sluggish respiratory response to ADP in liver mitochondria of alloxan diabetic rats and hibernating ground squirrels was overcome by addition of uncouplers of oxidative phosphorylation, suggesting that poor penetration of ADP prevented maximum oxygen consumption. The in vivo depression of adenine nucleotide translocation through the inner mitochondrial membrane was confirmed directly by measurements of [14C]ADP translocase and 32P-ATP exchange reactions. The inhibition of adenine nucleotide penetration was considered to be a consequence of increased hepatic lipid content, particularly long chain acyl-CoA esters, which occur in diabetes and hibernation. Demonstrated changes in the pattern of mitochondrial long chain fatty acids may be responsible for the altered spectral characteristics of the cytochromes observed in these animals. The inhibition of adenine nucleotide translocation could be reproduced in vitro with normal rat liver mitochondria by production of, or upon addition of certain esters of long chain fatty acids. Simultaneous addition of pyruvate or α-ketoglutarate with the free fatty acid prevented formation of the acyl-CoA ester and inhibition of translocation. Selective reversal of the inhibition by carnitine provided strong evidence implicating the acyl-CoA ester rather than the free fatty acid as the inhibitory agent. From these results, it is postulated that certain long chain acyl-CoA esters, acting as natural effectors of adenine nucleotide translocation, can perform a physiological role in the regulation of mitochondrial metabolism.

MATERIALS AND METHODS

Chemicals—Alloxan and d,l-carnitine hydrochloride were purchased from Schwarz-Mann; [14C]ADP from Schwarz Bio-Research; 32P inorganic phosphate from New England Nuclear; [14C]ATP and NCS Solubilizer from Amer sham-Searle; acyl-CoA derivatives from P-L Biochemicals; cytochrome c, type III, and bovine serum albumin-fatty acid-free, type F, from Sigma; and atractyloside from Calbiochem. Salicylanilide XIII, 5-Cl, 3-t butyl, 2'-Cl, 4'-NO2-salicylanilide, was kindly donated by Dr. Henry A. Lardy. All other reagents were of the highest grade commercially available.

In a previous report (1), we suggested that poor penetration of adenine nucleotides could account for the sluggish respiratory response observed in mitochondria of the hibernating ground squirrel. Subsequent studies conclusively showed that adenine nucleotide translocase activity could be inhibited in normal rat liver mitochondria upon addition of oleyl-CoA at concentrations too low to uncouple oxidative phosphorylation (2). These independent observations are consistent with the proposal that certain long chain fatty acid esters which accumulate in the liver during periods of excessive fatty acid oxidation act as natural regulators of adenine nucleotide translocase activity. Diabetes and hibernation, potent stimulants for fatty acid oxidation by the liver, were considered to be good model systems to test the validity of this hypothesis.

The present communication describes studies on adenine nucleotide translocation in liver mitochondria of the diabetic rat and monkey as well as the hibernating ground squirrel. Preliminary experiments restricted to the effects of oleic acid and its CoA ester on adenine nucleotide translocase activity of normal rat liver mitochondria have been confirmed and extended to include other fatty acids and acyl-CoA derivatives. The results, which provide evidence for inhibition of translocase activity in diabetes and hibernation, substantiate the conclusion that this inhibition is caused by certain long chain fatty acid esters can modulate reverse electron transport through transition from State 3 to State 4 respiration and effectively regulate mitochondrial metabolism.

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Madison, Wis.) weighing 180 to 200 g were maintained on a Purina Laboratory Chow diet and water ad libitum. Following an overnight fast, the rats were injected intravenously with 50 mg of alloxan per kg of body weight and were used approximately 3 to 5 days after injection when blood sugars ranged from 300 to 400 mg/100 ml and urinary ketones were positive. Alloxan diabetic Rhesus monkeys were maintained approximately 8 years before killing. During this time, the chronically glucosuric monkeys were maintained on daily NPH-insulin to prevent weight loss. Insulin was withheld 24 hours prior to killing at which time the animals became ketotic. We are indebted to Doctors Ronald Engerman and J. M. Bloodworth, Departments of Ophthalmology and Pathology, University of Wisconsin, Medical School, Madison, Wis., for liver samples from these animals, which are part of a continuing study of diabetic retinopathy. Ground squirrels (Citellus tridecemlineatus), weighing 250 to 280 g, were obtained during hibernation from the International Zoological Center, Hinsdale, Ill., and were housed individually in complete darkness at 0°C with water available ad libitum. Hibernation, which occurred in November, was allowed to continue approximately 3 months before the animals were killed. Active normothermic ground squirrels serving as controls were removed from the cold room after natural arousal and maintained for at least 1 month under normal conditions of feeding and temperature.

Preparation of Mitochondria—All animals were killed by decapitation and livers were removed as quickly as possible. The temperature for all further manipulations was maintained at 0-3°C unless otherwise stated. Mitochondria were prepared by differential centrifugation according to the method of Schneider (3), except that a medium containing 250 mM mannitol, 70 mM sucrose, and 1.0 mM Tris-EDTA (final pH 7.4) was used to homogenize the liver as well as suspend the mitochondria after washing them once with 250 mM sucrose.

Preparation of Crude Cytochrome Oxidase—Liver mitochondria were suspended in a solution of 50 mM Tris-HCl, pH 8.0, 660 mM sucrose, and 10 mM histidine at a protein concentration of 23 mg per ml. Following addition of 10% (w/v) potassium deoxycholate, pH 7.5 (0.3 mg per mg of protein), and solid KCl (7.2 g/100 ml), the suspension was stirred for 10 min at 0°C and centrifuged at 78,000 X g for 20 min in a Spinco model L Ultracentrifuge. After one wash, the pellet was resuspended in the original medium and used for the cytochrome oxidase assay.

Fatty Acid Analysis—Methyl esters of fatty acids from the mitochondrial lipids were prepared with BF₃-MeOH after hydrolysis with 0.5 N NaOH in methanol (4). Gas-liquid chromatography was performed with a F and M Biomedical gas chromatograph, model 400, equipped with a glass column, 0.32 X 183 cm, packed with 6% diethylene glycol succinate polyester on Chromosorb W (60 to 80 mesh). The instrument was operated isothermally at 165°C. The methyl esters were identified by comparing retention time with standards (National Institutes of Health Mixture D) and by calculating equivalent chain lengths. Quantitation was done by triangulation.

Assay Methods—Oxygen consumption and respiratory control ratios were measured polarographically as described by Estabrook (5), with a Clark oxygen electrode supplied with the Gilson Oxygraph. The ²³P-ATP exchange activity was determined by the method of Boyer et al. (6). Translocation of adenine nucleotides was determined by the method of Wojtczak and Zaluska (7) with the following modifications. The washed mitochondrial fraction was allowed to solubilize overnight in NCS reagent (quaternary ammonium base in 0.6 N Toluene), and then counted in 20 ml of Bray's scintillation fluid (8) with a Packard Tri-Carb scintillation spectrometer model 3314. Crude cytochrome oxidase preparations were measured spectrophotometrically by the method of Wharton and Tzagoloff (9), mitochondrial protein by the biuret method, (10) and hemoproteins were determined quantitatively as pyridine hemochromogens (11). Difference spectra for ground squirrel liver mitochondria were obtained directly with an Amino Chance dual wave length spectrophotometer and rat liver mitochondria with a Cary II spectrophotometer after initial treatment according to the method of Williams (12). Acetocetate was prepared according to the method of Seely (13).

RESULTS

Mitochondrial Respiration in Livers of Diabetic and Hibernating Animals—Initial studies compared respiratory control in liver mitochondria of diabetic and hibernating animals with their control counterparts. In contrast to the control, ADP added to mitochondria of the experimental animal respiring in the presence of substrate and inorganic phosphate, i.e. State 4, did not maximally stimulate oxygen consumption (Fig. 1). The depressed response to external addition of ADP was consistently more pronounced in the hibernator than in the diabetic animals. In the diabetic rat the depression which appeared to be directly proportional to the severity of the diabetes could be at least partially eliminated by extensively washing the mitochondria. In both the diabetic and the hibernator, the low oxygen consumption during the State 3 to 4 cycle was stimulated by the addition of Safypanilamide XIII, a potent uncoupler of oxidative phosphorylation (14). These results indicate that the mitochondria were not uncoupled but that the penetration of ADP through the inner mitochondrial membrane was decreased.

²³P-ATP Exchange Activity and [³¹C]ADP Translocation in Liver Mitochondria of Diabetic and Hibernating Animals—In order to examine the penetrability of the adenine nucleotides directly, [³¹C]ADP and [³¹C]ATP translocation experiments were performed. In addition, the ²³P-ADP exchange activity, an index of oxidative phosphorylation, was also measured. This exchange reaction, which is dependent upon the ability of exogenously supplied ATP to penetrate the inner mitochondrial membrane, should parallel that of adenine nucleotide translocation. Results of these experiments as shown in Tables I and II are consistent with the limited ADP stimulation of mitochondrial respiration in diabetic and hibernating animals. In all cases, ²³P-ATP exchange activity and [³¹C]ADP translocation were lower in the experimental groups. The more pronounced decrease exhibited by the diabetic monkey and hibernating ground squirrel could represent species difference, but is more likely a result of the intensity of the metabolic condition. Carnitine, which facilitates oxidation of acyl-CoA esters through the carnitine acyl transferase enzyme (or enzymes), and α-ketoglutarate, which by preferentially utilizing CoA through the α-ketoglutarate dehydrogenase complex prevents activation of endogenous fatty acids, revert the decreased ³²Pi-ATP exchange reaction, which is dependent upon the ability of exogenously supplied ATP to penetrate the inner mitochondrial membrane, should parallel that of adenine nucleotide translocation. Results of these experiments as shown in Tables I and II are consistent with the limited ADP stimulation of mitochondrial respiration in diabetic and hibernating animals. In all cases, ²³P-ATP exchange activity and [³¹C]ADP translocation were lower in the experimental groups. The more pronounced decrease exhibited by the diabetic monkey and hibernating ground squirrel could represent species difference, but is more likely a result of the intensity of the metabolic condition. Carnitine, which facilitates oxidation of acyl-CoA esters through the carnitine acyl transferase enzyme (or enzymes), and α-ketoglutarate, which by preferentially utilizing CoA through the α-ketoglutarate dehydrogenase complex prevents activation of endogenous fatty acids, revert the decreased ²³P-ATP exchange reaction and [³¹C]ADP translocase activities toward normal. However, restoration of [³¹C]ADP uptake was only marginal with the mitochondria from the hibernating squirrels. Cyanide,

These data are not shown, since they are identical with the results with [³¹C]ADP.
**TABLE I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Rat Normal</th>
<th>Rat Diabetic</th>
<th>Monkey Normal</th>
<th>Monkey Diabetic</th>
<th>Ground Squirrel</th>
<th>Active Ground Squirrel</th>
<th>Hibernate Ground Squirrel</th>
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<tbody>
<tr>
<td></td>
<td>cpm/mole ATP</td>
<td></td>
<td>cpm/mole ATP</td>
<td></td>
<td>cpm/mole ATP</td>
<td>cpm/mole ATP</td>
<td>cpm/mole ATP</td>
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<tr>
<td>None</td>
<td>452 ± 10.8</td>
<td>360 ± 8.2</td>
<td>320 ± 9.2</td>
<td>180 ± 5.4</td>
<td>332 ± 6.5</td>
<td>88 ± 2.5</td>
<td></td>
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<tr>
<td>dl-Carnitine, 5.0 mm</td>
<td>463 ± 11.2</td>
<td>443 ± 9.4</td>
<td>330 ± 8.5</td>
<td>290 ± 6.3</td>
<td>347 ± 9.5</td>
<td>129 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate, 5.0 mm</td>
<td>469 ± 12.0</td>
<td>432 ± 11.6</td>
<td>398 ± 9.5</td>
<td>264 ± 8.5</td>
<td>340 ± 15.1</td>
<td>151 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>KCN, 1.0 mm</td>
<td>341 ± 13.2</td>
<td>270 ± 7.3</td>
<td>280 ± 6.1</td>
<td>94 ± 10.1</td>
<td>285 ± 7.3</td>
<td>51 ± 1.5</td>
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</tr>
<tr>
<td>KCN + α-Ketoglutarate</td>
<td>445 ± 8.5</td>
<td>363 ± 11.0</td>
<td>315 ± 12.2</td>
<td>251 ± 4.8</td>
<td>320 ± 8.1</td>
<td>99 ± 4.8</td>
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* Standard error (five to six animals in each group).

**TABLE II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Rat Normal</th>
<th>Rat Diabetic</th>
<th>Monkey Normal</th>
<th>Monkey Diabetic</th>
<th>Ground Squirrel</th>
<th>Active Ground Squirrel</th>
<th>Hibernate Ground Squirrel</th>
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<td></td>
<td>cpm × 10⁴/mg protein</td>
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<td>cpm × 10⁴/mg protein</td>
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<td>cpm × 10⁴/mg protein</td>
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<tr>
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<td>392 ± 6.7</td>
<td>157 ± 21.4</td>
<td>260 ± 18.6</td>
<td>85 ± 9.5</td>
<td>267 ± 4.6</td>
<td>27 ± 1.8</td>
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<tr>
<td>dl-Carnitine, 5.0 mm</td>
<td>280 ± 2.0</td>
<td>255 ± 9.5</td>
<td>258 ± 17.1</td>
<td>168 ± 4.2</td>
<td>265 ± 9.1</td>
<td>55 ± 1.7</td>
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<tr>
<td>Albumin, 15 mg</td>
<td>270 ± 2.3</td>
<td>228 ± 10.4</td>
<td>261 ± 8.5</td>
<td>134 ± 7.1</td>
<td>266 ± 3.7</td>
<td>52 ± 1.7</td>
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* Standard error (five to six animals in each group).

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**Fig. 1.** Respiratory control and response to salicylanilide XIII by liver mitochondria from active and hibernating ground squirrels and normal and diabetic rats. Liver mitochondria (8.0 mg of protein) was added to a 2.0-ml reaction mixture containing 20 mM KCl, 225 mM sucrose, 10 mM KH₂PO₄, 5 mM MgCl₂, and 20 mM triethanolamine-HCl, pH 7.4. At the points indicated, 2.0 mM succinate was added followed by additions of 0.35 mM ADP and 0.2 mmol salicylanilide XIII. The numbers in the O₂ tracing represent the respiration rate expressed in micromoles of oxygen per mg of protein per hour. The oxygen concentration at the time of the succinate addition was 0.94 mm.

Previously reported by Boyer et al. (6) to minimally inhibit ATP-ATP exchange would appear to act in the present experimental system by preventing complete oxidation of endogenous long chain fatty acyl-CoA esters, which in turn decrease penetration of exogenously added ATP. The more intense inhibition upon addition of KCN, as well as reversal of the inhibition of α-ketoglutarate and albumin, tends to support this contention.

**Inhibition of ATP-ATP Exchange Activity and [¹⁴C]ADP Translocation in Normal Rat Liver Mitochondria by in Vitro Addition of Long Chain Fatty Acids and Their CoA Esters—**An in vitro system was developed with rat liver mitochondria to simulate the condition of increased hepatic fatty acid oxidation which occurs during hibernation and diabetes (9). ATP-ATP exchange activity and translocation of [¹⁴C]ADP were measured in rat liver mitochondria in the presence of fatty acids and cyanide at concentrations too low to produce uncoupling of oxidative phosphorylation and stimulation of ATPase activity. In the presence of KCN to permit accumulation of CoA esters, shorter chain fatty acids represented by butyric and octanoic acids had no effect on ATP-ATP exchange and [¹⁴C]ADP translocase activities. In contrast, myristic, palmitic, and oleic acids showed strong inhibition in the presence of KCN. α-Bromomyristic acid, which is a relatively poor substrate for fatty acid activation to the acyl-CoA ester (15), did not cause inhibition. Stearic and erucic, long chain saturated and unsaturated fatty acids, respectively, showed significant inhibition in the presence of KCN.
esters. As shown in Table IV, [14C]ADP translocation was inhibited 50 and 92% at concentrations of 3 and 30 μM, respectively, of either oleoyl- or myristoyl-CoA. That carnitine, but not α-ketoglutarate reversed the effect, even at the high concentration of acyl-CoA, localized the site of inhibition to the inner mitochondrial membrane. The inhibitory effect of stearoyl- and palmitoyl-CoA which was of lesser magnitude was also overcome by the addition of carnitine. Under identical experimental conditions the [32P]ATP exchange activity responded in

<table>
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<th>Exchange activity</th>
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<tbody>
<tr>
<td>None</td>
<td>415</td>
<td>276</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>410</td>
<td>273</td>
</tr>
<tr>
<td>Myristic acid + KCN</td>
<td>103</td>
<td>140</td>
</tr>
<tr>
<td>Myristic acid + α-ketoglutarate</td>
<td>398</td>
<td>287</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>438</td>
<td>283</td>
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<tr>
<td>Palmitic acid + KCN</td>
<td>256</td>
<td>100</td>
</tr>
<tr>
<td>Palmitic acid + α-ketoglutarate</td>
<td>449</td>
<td>287</td>
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<tr>
<td>Oleic acid</td>
<td>460</td>
<td>280</td>
</tr>
<tr>
<td>Oleic acid + KCN</td>
<td>93</td>
<td>104</td>
</tr>
<tr>
<td>Oleic acid + α-ketoglutarate</td>
<td>450</td>
<td>291</td>
</tr>
<tr>
<td>Oleic acid + pyruvate</td>
<td>398</td>
<td>287</td>
</tr>
<tr>
<td>Oleic acid + acetoacetate</td>
<td>101</td>
<td>106</td>
</tr>
<tr>
<td>Oleic (previously incubated) + KCN + α-ketoglutarate</td>
<td>106</td>
<td></td>
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<tr>
<td>Atractyloside</td>
<td>36</td>
<td>18</td>
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<tr>
<td>Atractyloside + α-ketoglutarate</td>
<td>411</td>
<td>22</td>
</tr>
<tr>
<td>Atractyloside + carnitine</td>
<td>33</td>
<td>20</td>
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reasons for this are unclear at present. The inhibitory effects by long chain fatty acids and cyanide on adenine nucleotide translocation were completely reversed by simultaneous addition of α-ketoglutarate or pyruvate (Table III). Preliminary incubation of the fatty acid (oleic) and KCN for 10 min prior to addition of the α-keto acid prevented the reversal. These results indicate that inhibition of translocase activity, due to activation of the fatty acids to their CoA esters, can be prevented by preferential utilization of endogenous CoA through the α-ketoglutarate and pyruvate dehydrogenase enzymes. An alternative possibility is that fatty acids selectively block electron transport and the block can be overcome by oxidation of NADH through lactate and glutamate dehydrogenase. However, the inhibition was not reversed by addition of acetoacetate, which is readily reduced to β-hydroxybutyrate in liver mitochondria. The acetoacetate was tested with purified P-hydroxybutyrate dehydrogenase before use. The inhibition by atracyloside, which has a potent effect on adenine nucleotide translocation (16), cannot be overcome by α-keto acids or carnitine.

Studies were next undertaken to examine the effect of acyl-CoA esters directly on [32P]ATP exchange and translocation of adenine nucleotides. The translocation experiments were performed at 0° to prevent complete oxidation of the acyl-CoA esters. As shown in Table IV, [32P]ATP translocation was inhibited 30 and 92% at concentrations of 3 and 30 μM, respectively, of either oleoyl- or myristoyl-CoA. That carnitine, but not α-ketoglutarate reversed the effect, even at the high concentration of acyl-CoA, localized the site of inhibition to the

![Fig. 2. Difference spectra for the cytochrome components of active and hibernating ground squirrel liver mitochondria. Mitochondrial protein from active ground squirrel, 27 mg, and from hibernator, 37.5 mg were diluted to 3.0 ml with 0.25 M sucrose and 0.05 M Tris-HCl, pH 7.4. Oxidized (potassium ferriyvanide) minus reduced (sodium hydrosulfite). Note-The fourth number from the left on the abscissa should be 500 rather than 500.](http://www.jbc.org/)

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a parallel manner. Reversal of long chain acyl-CoA ester inhibition by carnitine is more complete in mitochondria from normal animals than those from the diabetic and hibernators. Livers from these animals contain high concentrations of endogenous acyl-CoA esters which may not be completely converted to acyl carnitine. Guinea pig liver and rat heart mitochondria responded similarly to addition of long chain acyl-CoA esters, indicating a rather general species and tissue effect.

**Difference Spectra and Cytochrome Oxidase Activity in Liver Mitochondria** In a previous study (1) we observed a striking alteration of the cytochrome spectra from hibernating ground squirrel mitochondria. In order to verify this interesting and somewhat surprising phenomena (Fig. 2), special care was taken to allow a sufficient number of control animals to become active after hibernation. Studies were also carried out on diabetic and normal rat liver mitochondria (Fig. 3) to determine whether the spectral changes were related to factors common to the metabolism of both hibernation and diabetes. In the diabetic rat as well as the hibernating ground squirrel mitochondria, the peak height of a-a3 decreased compared with control animals. The spectral change occurred consistently in liver mitochondria from all hibernators tested, whether Citillus lateralis or C. tridecemlineatus and was never observed in animals allowed to awaken from hibernation and become active. The extent of the reduction of the a-a3 peak height was much greater in the hibernator than in the diabetic. However, the spectral changes in diabetic rat liver mitochondria were not always observed and were most evident in severely ketotic animals. Following treatment with insulin, diabetic rats always had normal spectra. Difference spectra (not shown) of heart mitochondria from diabetic and normal rats were identical. Pyridine hemochromogen determinations for total mitochondrial cytochromes revealed an average value for the hibernator which was half that for the active animal. Although values for the diabetic were lower than normal, the differences were not significant. The specific activity of cytochrome oxidase assayed enzymatically was lower in both the hibernator and diabetic, although again the extent of the decrease was much greater in the hibernator (Table V).

**Fatty Acid Patterns in Liver Mitochondria from Hibernating and Diabetic Animals**—While the functional significance is not completely understood, the altered cytochrome spectra might reflect changes in structural components of liver mitochondrial membranes brought about by abnormal fatty acid and phospholipid metabolism. The fatty acid patterns of liver mitochondria from ground squirrels and rats are shown in Table VI. Diabetic rat liver mitochondria contain a higher percentage of palmitic acid and lower percentages of arachidonic acid and total polyunsaturated acids as compared to the normal control rat. This result is consistent with a defect in the oxidative de
steady state of phosphorylation, the rate of ATP formation in-
Acknowledgments—We wish to express our appreciation to Professor H. A. Lardy for his continued interest and support.

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Corrections

Vol. 247 (1972) 1513-1519

In Lerner, Edith, Austin L. Shug, Charles Elson, and Earl Shrago. Reversible Inhibition of Adenine Nucleotide Translocation by Long Chain Fatty Acyl Coenzyme A Esters in Liver Mitochondria of Diabetic and Hibernating Animals

The date received for publication was Oct. 12, 1971, not Oct. 12, 1970.

Vol. 246 (1971) 6019-6023

In Marshall, Thomas H., and Ay Agkun. The Specificity of Porcine Elastase and \( \alpha \)-Chymotrypsin. Effect of Fatty Acid Chain Length in a Homologous Series of Nitrophenyl Esters

Page 6021, Table I, the third entry under “Chymotrypsin-catalyzed hydrolysis at pH 7.8, 25°C” should read:

\[
\begin{array}{cccc}
\text{n-Butyrate} & 13 & 0.711 & 0.625 \\
\end{array}
\]

Vol. 247 (1972) 2095-2101


Page 2099, Equation 5 should have a superscript 2 outside the brackets

\[
\frac{1}{E_0} = \left( \frac{k_2}{k_2 + k^3} \right)^2
\]

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