# Influence of Streptozotocin Diabetes on Intestinal 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in the Rat

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SUMMARY

**Studies were undertaken to examine cholesterogenesis in the intestine of streptozotocin-diabetic rats by measuring incorporation of [2-$^{14}$C] acetate into cholesterol and 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase, EC 1.1.1.34) activity. In these diabetic rats, the intestinal mucosal weight and food consumption were markedly high. The incorporation of [2-$^{14}$ C] acetate into cholesterol was significantly increased in all diabetic intestinal segments. However, the rates of production of fatty acids and carbon dioxide were not affected. Hepatic HMG-CoA reductase activities were markedly reduced during both the diurnal high and low periods in these diabetic rats, and there was no diurnal variation. In contrast, the specific activities of this enzyme in jejunal crypt cells during both the diurnal high and low periods were significantly higher in these diabetic rats without loss of diurnal variation. Total reductase activity per segment of intestine in jejunal and ileal mucosa (villi + crypt cells) was increased in these diabetic rats. Control rats had higher total and specific activity of ileal mucosal (villi + crypt cells) reductase than of jejunal mucosal reductase during the diurnal high period. The jejunal-ileal gradient in reductase activity and the incorporation of [2-$^{14}$ C] acetate into cholesterol did not change significantly with streptozotocin-diabetic rats. The results indicate that in streptozotocin-diabetic rats, hepatic cholesterogenesis decreases but intestinal synthesis increases.** DIABETES *26*:439-44, May, 1977.

3-Hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase, EC 1.1.1.34) is considered to be the rate-controlling enzyme of cholesterol biosynthesis in the liver and the intestine.[1] However, intestinal cholesterogenesis has not been worked out in such detail as in the liver. Recent studies have shown that the microsomal HMG-CoA reductase of the liver and the intestinal mucosa have similar kinetic characteristics and pH-optima.[2] It is suggested that in the baboon the liver is the predominant source of plasma cholesterol during low cholesterol intake, contributing about 75 per cent of the total daily production.[3] During high cholesterol intake, hepatic synthesis is suppressed to about 25 per cent of the control rate, whereas ileal synthesis is still more than half the control rate and exceeds the hepatic rate by about 100 per cent (per gram of tissue).[3] Recent studies have shown that the activity of the hepatic HMG-CoA reductase differs with the age and the sex of the experimental animals while that of the intestinal mucosa does not.[2] Thus, the accumulated evidence indicates that there may be a difference in regulatory mechanisms between the hepatic and the intestinal cholesterogenesis.[1,5-9]

In diabetic rats, hepatic HMG-CoA reductase activity and its diurnal variation are reduced to extremely low levels as compared with normal animals.[10] Insulin produces a rapid stimulation of the enzyme activity in the diabetic rat to the levels found in normal animals at that time of day.[10] Although these studies have contributed significantly to our understanding of cholesterogenesis in diabetes, only limited data are available concerning intestinal cholesterogenesis in this disorder. The studies reported here were designed

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to investigate cholesterogenesis in the diabetic rat intestine by measuring HMG-CoA reductase activity and the rates of acetate incorporation into cholesterol.

## MATERIALS AND METHODS

[$2^{-14}$C] sodium acetate (50 mCi./mmole) and D,L-3-hydroxy-3-methyl [$3^{-14}$ C] glutaryl CoA (18.5 mCi./mmole) were purchased from New England Nuclear (Boston, Mass.). Unlabeled D,L-HMG-CoA was obtained from P-L Biochemicals (Milwaukee, Wis.). Unlabeled D,L-mevalonic acid lactone, glucose 6-phosphate dehydrogenase, NADP, dithiothreitol, EDTA, sodium acetate, and digitonin were obtained from Sigma (St. Louis, Mo.).

Male Wistar rats weighing 200 to 250 gm. were maintained ad libitum on water and a standard rat chow (Oriental Yeast, Tokyo). Rats were housed in a windowless, air-conditioned room that was artificially illuminated from 7 a.m. to 7 p.m. Diabetes was induced by an intravenous injection of 65 mg. streptozotocin (Upjohn, Kalamazoo, Mich.) per kilogram body weight in 0.05 M citrate buffer (pH 4.5). The animals were killed by cervical dislocation four to five weeks later. The mean daily food consumption of diabetic and control animals was measured for two weeks prior to sacrifice.

Gastrointestinal tracts were rapidly removed, rinsed with cold saline, and placed in cold Krebs' bicarbonate buffer (pH 7.4) after sacrifice. A 3-cm. segment of intestine, measured at either 10 cm. from the pylorus (jejunum), the middle of the intestine (midintestine), or 1 cm. from the ileocecal junction (ileum), was placed on a cold glass plate.[11] These intestinal segments were thoroughly rinsed with cold buffer solution and blotted on filter paper. Slices 1 mm. thick were prepared from each segment level, and 400 mg. of these slices was then placed in incubation flasks (fitted with center well) containing 5 ml. of Krebs' bicarbonate buffer (pH 7.4), 2.5 $\mu$Ci. of [$2^{-14}$C] sodium acetate, and 10 $\mu$moles of sodium acetate. The assays for conversion of [$2^{-14}$C] acetate into cholesterol, fatty acids, and carbon dioxide described by Dietschy and Siperstein[1,7] were adapted for these experiments, with the exception that the carbon dioxide derived was trapped in 0.5 ml. hyamine hydroxide previously placed in the center well of each flask. The data are expressed as nmoles of [$2^{-14}$C] acetate incorporated into cholesterol, fatty acids, and carbon dioxide per gram wet weight of tissue per two hours' incubation.

The livers were removed immediately after cervical dislocation and chopped with sterile blades before homogenization. All subsequent operations were carried out at 0-5° C. A 1-gm. aliquot was homogenized in a Potter-Elvehjem homogenizer with 9 ml. of a solution (pH 7.2) containing 0.3 M sucrose, 0.075 M nicotinamide, 0.002 M EDTA, and 0.02 M mercaptoethanol.[12] The homogenate was centrifuged for 10 minutes at 12,000 g in a refrigerated centrifuge (4° C.), and the fluffy layer at the top of the tube was carefully discarded by use of a Pasteur pipette. The supernatant microsomal fraction was then centrifuged at 105,000 g for 60 minutes at 4° C.[13] The sedimented microsomal pellet was then homogenized in the centrifuge tube with the above homogenizing medium.

A 15-cm. segment of intestine measured at 10 cm. from the pylorus (jejunum) was taken for crypt cells. Preparation of crypt cells confirmed histologically was made as described by Dietschy and Siperstein.[7] A 3-cm. segment of intestine, measured at either 10 cm. from the pylorus (jejunum) or 1 cm. from the ileocecal junction (ileum), was taken for total mucosa. The total mucosa was collected by gentle scraping with a glass slide and then weighed.[11] The microsomal fractionation of the cell fractions was carried out as described above for the liver, but a 5 per cent homogenate was prepared for the crypt-cell fraction.

The final composition of HMG-CoA reductase assay system was determined on the basis of experiments dealing with the effects of pH, substrate, and microsomal protein concentration and the time course of the reaction by using intestinal crypt cells from normal animals. The complete assay system came to a total volume of 0.1 ml.: 100 mM phosphate buffer (pH 7.2), 3 mM NADP, 10 mM neutralized EDTA, 30 mM glucose 6-phosphate, 0.35 U. glucose 6-phosphate dehydrogenase, 10 mM dithiothreitol, 0.14 mM D,L-[$3^{-14}$C] HMG-CoA, and 0.1-0.5 mg. microsomal protein. The assays were started by the addition of microsomal protein. Incubations were terminated by adding 10 $\mu$l. of 10 N NaOH after 20 minutes at 37° C. Then 10 $\mu$l. of 20 $\mu$moles mevalonic acid lactone was added, and the samples were incubated at 37° C. for 10 minutes. After addition of 20 $\mu$l. of concentrated HCl, the samples were further incubated at 37° C. for 60 minutes to permit mevalonic acid to lactonize. It was then centrifuged three minutes to sediment denatured protein out. Fifty-microliter portions of the protein-free supernatant solution were directly applied to plastic-backed $F_{254}$ silica gel thin-layer sheets (E. Merk, Darmstadt).

The chromatograph was developed in benzene-acetone (1:3, V/V) and air-dried. The average Rf value of mevalonic acid lactone was 0.48. The position of the mevalonic acid lactone band was marked under ultraviolet illumination and confirmed by radio thin-layer chromatogram scanning. A strip corresponding to the Rf of the mevalonic acid lactone was cut and placed in a scintillator[13] and the radioactivity was assayed by an Aloka LSC 651 liquid scintillation counter. Quenching was corrected by the external-standard method. Raw $^{14}C$ data are corrected for recovery by use of [$^{3}H$] mevalonic acid lactone as the internal standard.[15] Corrected recovery averaged 93 per cent. Enzyme activities are expressed as pmoles mevalonic acid lactone formed per milligram protein per minute.

Serum insulin was determined by a radioimmunoassay method.[16,17] Serum cholesterol was measured by the method of Zak et al.[18,19] Blood glucose was determined in an AutoAnalyzer by a ferricyanide method.[20] Protein concentration was determined with Folin phenol reagent.[14] The significance of the results was calculated by Student's *t*-test.[21]

## RESULTS

As shown in table 1, the mucosal wet weight of diabetic rats was 49 per cent greater in the jejunum and 39 per cent greater in the ileum than in the control segments. Food consumption of the diabetic animals was 84 per cent greater during dark periods and 41 per cent greater during light periods. Thus, both mucosal wet weight and food consumption were markedly increased in the diabetic rat.

Serum insulin levels in diabetic rats decreased from 28.3 ± 2.1 to 11.5 ± 1.5 μU./ml. at noon and decreased from 25.9 ± 1.5 to 11.4 ± 1.1 μU./ml. at midnight (table 1). There was no difference between noon and midnight serum insulin levels of the control animals. Serum total cholesterol levels in diabetic rats increased from 72.2 ± 2.8 to 85.6 ± 4.2 mg./100 ml. at noon and increased from 62.3 ± 2.7 to 79.7 ± 3.8 mg./100 ml. at midnight (table 1). Serum total cholesterol levels were increased slightly but significantly in the diabetic rat.

As shown in table 2, the incorporation of [$2^{-14}C$] acetate into digitonin precipitate sterols (cholesterol) in the jejunum of diabetic rats increased from 11.8 ± 1.3 to 19.8 ± 1.8 nmoles per gram of wet weight tissue per two hours ($p < 0.01$). In the midintestine it increased from 21.7 ± 4.8 to 35.7 ± 7.1 ($p < 0.02$). In the ileum it increased from 218.1 ± 27.6 to 357.4 ± 18.1 ($p < 0.001$). These results indicate that the incorporation of [$2^{-14}C$] acetate into cholesterol increases significantly in all diabetic intestinal segments. In addition, the jejunal-ileal gradient in the intestinal cholesterogenesis persisted in the diabetic intestine. On the other hand, it did not affect the rate of fatty acid or carbon dioxide production except in the jejunum.

As shown in table 3, the specific reductase activity during the diurnal high period in the diabetic liver decreased from 204.0 ± 21.3 to 35.8 ± 8.3 pmoles of mevalonic acid lactone formed per milligram protein per minute ($p < 0.001$). During the diurnal low period it decreased from 60.8 ± 10.2 to 25.7 ± 3.6 ($p < 0.02$). These results indicate that hepatic

TABLE 1

Metabolic data from control and streptozotocin-diabetic animals

| | Control | Diabetes | P value |
|---|---|---|---|
| Blood glucose (mg./100 ml.) | 112.2±4.1(13) | 383.9±24.3(14) | $p < 0.001$ |
| Mucosal wet weight (mg./cm.) | | | |
| Jejunal mucosa | 50.0±2.5 (8) | 74.6± 4.3 (8) | $p < 0.001$ |
| Ileal mucosa | 38.3±2.7 (8) | 53.2± 2.8 (8) | $p < 0.01$ |
| Food consumption (gm.) | | | |
| 9 a.m.—9 p.m. (12 hours) | 9.0±0.5 (8) | 16.6± 0.9 (8) | $p < 0.001$ |
| 9 p.m.—9 a.m. (12 hours) | 16.7±0.4 (8) | 23.5± 0.6 (8) | $p < 0.001$ |
| 9 a.m.—9 a.m. (24 hours) | 25.7±0.7 (8) | 40.1± 1.2 (8) | $p < 0.001$ |
| Serum cholesterol (mg./100 ml.) | | | |
| Noon | 72.2±2.8(11) | 85.6± 4.2(11) | $p < 0.02$ |
| Midnight | 62.3±2.7(11) | 79.7± 3.8(14) | $p < 0.01$ |
| Serum insulin (μU./ml.) | | | |
| Noon | 28.3±2.1(10) | 11.5± 1.5(12) | $p < 0.001$ |
| Midnight | 25.9±1.5(14) | 11.4± 1.1(14) | $p < 0.001$ |

Mean ± standard error. Numbers in parentheses are number of rats used.
Noon: 1200. Midnight: 2400.

TABLE 2

Effects of streptozotocin diabetes on intestinal cholesterogenesis
([$2^{-14}$ C] acetate incorporated into cholesterol, fatty acids, and carbon dioxide)

| Products/tissues | Control | Diabetes | P value |
|---|---|---|---|
| Cholesterol | | | |
| Jejunum | 11.8± 1.3(10) | 19.8± 1.8(8) | $p < 0.01$ |
| Midintestine | 21.7± 4.8 (7) | 35.7± 7.1(8) | $p < 0.02$ |
| Ileum | 218.1± 27.6 (6) | 357.4± 18.1(8) | $p < 0.001$ |
| Fatty acids | | | |
| Jejunum | 76.9± 13.5 (8) | 146.6± 19.8(7) | $p < 0.02$ |
| Midintestine | 233.9± 29.8 (8) | 262.6± 61.3(6) | N.S. |
| Ileum | 278.8± 28.9 (7) | 335.7± 20.3(6) | N.S. |
| Carbon dioxide | | | |
| Jejunum | 2,063.2±200.1(11) | 3,094.8±232.3(8) | $p < 0.01$ |
| Midintestine | 3,974.3±315.4 (9) | 5,223.3±794.6(8) | N.S. |
| Ileum | 6,950.4±458.0(11) | 6,866.4±651.4(9) | N.S. |

Mean ± standard error. Numbers in parentheses are number of rats used. Experiments were done at noon (1200). The data are expressed as nmoles of [$2^{-14}$ C] acetate incorporated into cholesterol, fatty acids, and carbon dioxide per gram wet weight tissue during two-hour incubation period.

HMG-CoA reductase activity is extremely low in the diabetic rats. The diurnal rise was almost nonexistent in the diabetic liver. In contrast, the specific reductase activities during the diurnal high period in jejunal crypt cells of diabetic rats increased from 98.0 ± 17.1 to 167.6 ± 17.2 ($p < 0.02$). During the diurnal low period the activity increased from 46.2 ± 4.8 to 97.6 ± 13.1 ($p < 0.01$). These results indicate that the specific reductase activities in jejunal crypt cells during both the diurnal high and low periods increase significantly in the diabetic rats. In addition, the diurnal rise was observed in the diabetic intestine. Not only did the specific reductase activity in jejunal crypt cells increase during both the diurnal high and low periods in the diabetic animals, but also total activity per segment of intestine in the jejunal and ileal mucosa (villi + crypt cells) during the diurnal high period increased. As shown in table 4, the total reductase activity per centimeter of intestine during the diurnal high period in the jejunal mucosa (villi + crypt cells) of the diabetic rat increased from 31.7 ± 5.5 to 54.5 ± 6.7 pmoles of mevalonic acid lactone formed per centimeter of intestinal segment per minute ($p < 0.05$). In the ileal mucosa, total reductase activity increased from 88.9 ± 11.2 to 131.2 ± 6.5 ($p < 0.01$). In these diabetic animals there was no significant increase in specific reductase activities of the jejunal and ileal mucosa (villi + crypt cells) during the diurnal high period (table 5). In these diabetic and control animals, it was clearly shown that the ileal mucosa had higher total and specific activities of the reductase than those of jejunal mucosal reductase during the diurnal high period (tables 4 and 5). The jejunal-ileal gradient of the reductase activity did not change significantly with streptozotocin diabetes during the diurnal high period (tables 4 and 5).

TABLE 3

Effects of streptozotocin diabetes on HMG-CoA reductase activity in liver and intestine

| Tissues | Control | Diabetes | P value |
|---|---|---|---|
| Jejunal crypt cells | | | |
| Noon | 46.2± 4.8(6) | 97.6±13.1 (8) | $p < 0.01$ |
| Midnight | 98.0±17.1(7) | 167.6±17.1(10) | $p < 0.02$ |
| P value | $p < 0.05$ | $p < 0.01$ | |
| Liver | | | |
| Noon | 60.8±10.2(7) | 25.7± 3.6(11) | $p < 0.02$ |
| Midnight | 204.0±21.3(9) | 35.8± 8.3 (9) | $p < 0.001$ |
| P value | $p < 0.001$ | N.S. | |

Mean ± standard error. Numbers in parentheses are number of rats used. Noon: 1200. Midnight: 2400. The data are expressed as pmoles mevalonic acid lactone formed per milligram protein per minute.

## DISCUSSION

3-Hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) is considered to be the rate-

TABLE 4

Effects of streptozotocin diabetes on HMG-CoA reductase activity in total intestinal mucosa (villi + crypt cells)
(Total activity/cm. of intestinal segment)

| Tissues | Control | Diabetes | P value |
|---|---|---|---|
| Jejunum | 31.7± 5.5(8) | 54.5±6.2(7) | $p < 0.05$ |
| Ileum | 88.9±11.2(7) | 131.2±6.5(8) | $p < 0.01$ |
| P value | $p < 0.001$ | $p < 0.001$ | |

Mean ± standard error. Numbers in parentheses are number of rats used. Experiments were done at midnight (2400). The data are expressed as pmoles mevalonic acid lactone formed per centimeter of intestinal segment per minute.

TABLE 5

Effects of streptozotocin diabetes on HMG-CoA reductase activity in total intestinal mucosa (villi + crypt cells)

| Tissues | Control | Diabetes | P value |
|---|---|---|---|
| Jejunum | 94.5±17.3(8) | 128.0±13.7(7) | N.S. |
| Ileum | 226.4±23.2(7) | 241.1± 5.3(8) | N.S. |
| P value | $p < 0.001$ | $p < 0.001$ | |

Mean ± standard error. Numbers in parentheses are number of rats used. Experiments were done at midnight (2400). The data are expressed as pmoles mevalonic acid lactone formed per milligram protein per minute.

controlling enzyme of cholesterol biosynthesis in the liver and the intestinal mucosa.[1] However, intestinal cholesterogenesis has not been worked out in as much detail as in the liver. Recent studies have shown that the microsomal HMG-CoA reductases of liver and intestinal mucosa have similar kinetic characteristics and pH optima.[2] However, it is well known that there may be a difference in regulatory mechanisms between hepatic and intestinal cholesterogenesis.[1,5-9] In the diabetic rat, hepatic HMG-CoA reductase activity is reduced to extremely low levels compared with normal animals.[10] Cholesterogenesis in the diabetic intestine is therefore of interest. Previous studies in the rat have also shown that HMG-CoA reductase activities in the liver and the intestine have diurnal rhythmicity.[22] The diurnal rise in the level of hepatic HMG-CoA reductase activity is associated with feeding periods and can actually be altered by changing feeding times.[23] Although food consumption increases throughout the day in streptozotocin-diabetic rats, hepatic HMG-CoA reductase activity is reduced to low levels and there is almost no diurnal rise.[10] However, since diabetic animals are undoubtedly losing calories in the form of glucose in urine, the incremental change in body weight is not the same in the two types of animals.[33] The administration of insulin to normal or diabetic rats during the low point of the diurnal cycle caused a marked and rapid stimulation of HMG-CoA reductase activity in the liver.[10,24] Although we failed to demonstrate higher insulin levels at midnight, normal animals might have a point of higher insulin levels during dark periods associated with much more feeding. Recent studies have shown that there is no absolute requirement for insulin in the regulation of the reductase. For example, cholestyramine feeding can markedly stimulate this reductase activity in the liver of insulin-depleted rats.[25] In contrast to the low diabetic hepatic reductase activity, our results indicate that the intestinal reductase activity increases in diabetic rats without loss of diurnal variation. It seems likely that mechanisms of daily rises in hepatic and intestinal reductase activities are not regulated by the same factor(s) under these conditions. A high cholesterol diet in man and animals markedly suppresses hepatic cholesterogenesis but intestinal cholesterogenesis is not suppressed.[3,4] Furthermore, in the liver, reductase activity is located exclusively in the microsomal fraction.[2] In contrast, in the intestinal mucosa, reductase activity is found in both mitochondrial and microsomal fractions of crypt cells.[2] In addition, the activity of the hepatic enzyme differs with the age and sex of the experimental animals while that of the intestinal crypt cells does not.[2] Microsomal HMG-CoA reductases of the liver and intestinal mucosa have similar kinetic characteristics and immunologic reactivity.[2,34] These facts are not direct evidence that the enzymes from the two sources are identical.

Intestinal mucosal cholesterogenesis is mainly found in crypt cells but not in villi cells.[7] Therefore, the intestinal mucosal reductase activity is located only in the crypt cells.[2] The height of the villous mucosa also increases significantly after diabetes has progressed.[26,27] This might explain why we observed no change in specific activity of HMG-CoA reductase in total mucosa (villi + crypt cells) between normal and diabetic animals. Since HMG-CoA reductase is predominantly located in the crypt cells, the specific activity of the crypt enzyme would be reduced by the large contribution of villous protein; the specific activity of the enzyme increased, as measured in jejunal crypt mucosa after removal of the villous layer. In the diabetic animals, there was a significant increase in both mucosal wet weight and total enzymatic activity per centimeter of intestine. This increment was most striking in the jejunum, but it was also present in the ileum. In rats and monkeys, there is direct evidence that the intestinal wall contributes to serum cholesterol,[28,29] and it has been shown that in these species the intestine is the extrahepatic tissue exhibiting the highest rate of cholesterol synthesis.[5,6] Previous studies have also shown that in the rat the rate of hepatic cholesterol synthesis is controlled by the cholesterol in intestinal lymph lipoproteins.[30] Also, net cholesterol uptake from circulating intestinal lipoproteins takes place only in the liver, and only this tissue shows inhibition of cholesterol synthesis.[31] Therefore, it does seem appropriate to regard the intestine not only as a source of serum cholesterol but also as an indirect determinant of the rate of hepatic cholesterol synthesis.[32] It might be supposed that an accelerated cholesterol synthesis in the diabetic rat

intestine is an important contributing source of serum cholesterol and plays a greater role in suppression of hepatic cholesterol synthesis.

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