Induction of Diabetes in Animals by Parenteral Administration of Ferric Nitrilotriacetate

A Model of Experimental Hemochromatosis

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Rats and rabbits parenterally treated with a large daily dose of ferric nitrilotriacetate manifested diabetic symptoms such as hyperglycemia, glycosuria, ketonemia, and ketonuria after approximately 60 days of treatment. The blood insulin response to oral glucose loading was poor. Heavy iron deposits were found in liver parenchymal cells and in pancreatic exocrine cells, although some iron was deposited in the macrophages and reticuloendothelial cells of these organs. Faint iron staining was found in some pancreatic islet cells, with a reduction in beta granules and weak zinc staining. Cirrhotic liver changes and skin pigment deposition were not observed. Repeated blood withdrawals from ferric-nitrilotriacetate-treated animals resulted in disappearance of hyperglycemia, glycosuria, ketonemia, and ketonuria; disappearance of iron from the liver and pancreas; and restoration of islet beta granules to the control level. (Am J Pathol 95:663–674, 1979)

IDIOPATHIC HEMOCHROMATOSIS is characterized by symptoms of "bronze diabetes," ie, diabetes mellitus, generalized heavy iron deposits, skin pigmentation, and liver cirrhosis.[1-3] It is generally believed that this condition is due to disturbances in iron metabolism. Attempts have been made to induce experimental hemochromatosis by loading a variety of iron compounds, eg, colloidal iron,[4] saccharated iron oxide,[5-7] iron dextran,[5,8] and red blood cells,[7,9] in dogs, rats, and guinea pigs for periods ranging from 4 weeks to 7 years.[6] These iron compounds were administered by intravenous,[6] intraperitoneal,[4,10] and intramuscular [5,8] routes. None of these agents produced a satisfactory hemochromatosis.

Brown and associates [5] gave massive doses of iron (2.5 to 3.3 g/kg body weight to dogs. The animals showed severe symptoms of iron intoxication, eg, anorexia, apathy, and weight loss, but no evidence of cirrhosis or diabetes resembling idiopathic hemochromatosis was found. Cardiac function and glucose tolerance remained normal. Hepatic function was also normal, except for low serum protein concentrations. Death followed after 5 to 10 months of iron loading. Autopsies revealed slight changes in

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liver morphology, and the general histologic picture was that of hemosiderosis, not of hemochromatosis.

Hepatic cirrhosis without diabetes was induced in dogs by injecting a large amount of iron compounds: iron dextran intravenously and iron sorbitol intramuscularly.[11]

In our earlier experiments with colloidal iron administered parenterally,[12] the iron was selectively phagocytized by reticuloendothelial cells and was barely taken up by parenchymal cells. More recently, we reported on some preliminary diabetic changes after intraperitoneal administration of iron in the form of ferric nitrilotriacetic acid complex (Fe^{3+}-NTA).[13] In this paper the development of the diabetic process in parenterally iron-loaded rats and rabbits is reported.

Materials and Methods

Experimental Groups

A total of 224 inbred Wistar rats and 12 albino adult rabbits was used. The rats were divided into four major groups: Group I included animals treated with large doses of Fe^{3+}-NTA and their controls. Group II animals were used for examining hourly variations in serum iron levels after a single injection of Fe^{3+}-NTA. Group III animals were used for assessing the effects of blood depletion after Fe^{3+}-NTA overload. Group IV animals were used for observations of fibrotic changes in liver and pancreas after long-term, lethal injections of Fe^{3+}-NTA.

Group I (N = 120) rats were divided into four subgroups of 30 each: Group Ia rats received daily intraperitoneal (i.p.) injections of Fe^{3+}-NTA in the following sequence: 0.2 mg Fe/100 g body weight daily for 3 weeks, 0.6 mg Fe/100 g body weight daily for the next 3 weeks, and 1.0 mg Fe/100 g body weight daily for the remaining 2 months. The total amount of iron administered to each animal was approximately 200 mg. Group Ib rats were injected i.p. for the same duration with the same concentration of disodium nitrilotriacetic acid (Na_2-NTA) as Group Ia animals (NTA controls). Group Ic animals received equivalent injections of iron in colloid form (ferric hydroxide chondroitin sulfate colloid, Dainihon Pharmaceutical, Osaka)[12] as Group Ia animals. Group Id rats were untreated controls. Blood and urinary glucose and ketones were measured once weekly. After manifestation of glucosuria, urinary glucose was measured twice weekly in the morning. Animal body weight was measured twice weekly. Histologic studies were conducted after sacrifice.

Group II rats (N = 56) received one i.p. injection of Fe^{3+}-NTA at 1.0 mg Fe/100 g body weight. Blood was collected from the frontal orbital sinus at different intervals after injection. Serum iron concentration and the total iron-binding capacity were measured.

Group III rats (N = 24) received daily Fe^{3+}-NTA injections in the same schedule as Group Ia animals. After 2 months of iron loading, 2 ml of blood was withdrawn from the frontal orbital sinus once weekly for 4 weeks.

Blood and urinary sugar were measured twice weekly. At 5 weeks after termination of Fe^{3+}-NTA treatment, the animals were killed. Histochemical investigations of organ iron and beta-granules in pancreatic islet cells were performed.

Group IV rats (N = 24) received daily injections of Fe^{3+}-NTA by extending the schedule of Group Ia animals until death. Histologic observations were conducted on the tissue for cirrhotic changes.

The rabbits were used to confirm the ability of Fe^{3+}-NTA injections to induce diabetes as seen in the rats. Group A (N = 6) rabbits received Fe^{3+}-NTA injections i.p. at doses of 1.0 mg Fe/100 g body weight daily for 2 months. These rabbits and the controls (Group B, N = 6) were killed by severing the carotid artery. Each pancreas was removed and fixed with pure ethanol or Bouin's solution, and zinc was observed histochemically by the Okamoto method.[14]

Preparation of Fe^{3+}-NTA Solution

Ferric nitrate was dissolved in 1.0 N HCl. To produce Fe^{3+}-NTA solution, 162 ml of a 0.1 M ferric nitrate solution was added to 100 ml of 0.08 M disodium nitrilotriacetate (Eastman Kodak) solution, and the pH was adjusted to 7.4 with sodium bicarbonate powder under magnetic stirring.[15,16] The mixture was prepared immediately before use.

Serum Iron and Iron-Binding Protein

Serum iron (SI) concentrations and total iron-binding capacity (TIBC) were measured with the Fe Kit (Nippon Shoji Co., Osaka). Two solutions were used to check for the possible association of Fe^{3+}-NTA with serum proteins in blood: a) 0.05 M Tris-HCl buffer at pH 7.4 and b) a mixture of Tris buffer and 0.05 M Na_2-NTA at pH 7.4. The serum was eluted through a Sephadex G-15 column (2-cm diameter and 35-cm length). The proteins in the eluted samples were measured by the Biuret method.

Glucose and Ketones in Blood and Urine

Glucose concentrations in serum and urine were estimated using Ames Dexter Dexterstick (Miles Laboratory, Elkhart Indiana)[17,18]; occasional, more accurate measurements were made with Hoffman's[19] method and in some samples by the method devised by Lowry and associates.[20] Ketone levels were measured with Ames Ketostick.[21]

Serum Insulin

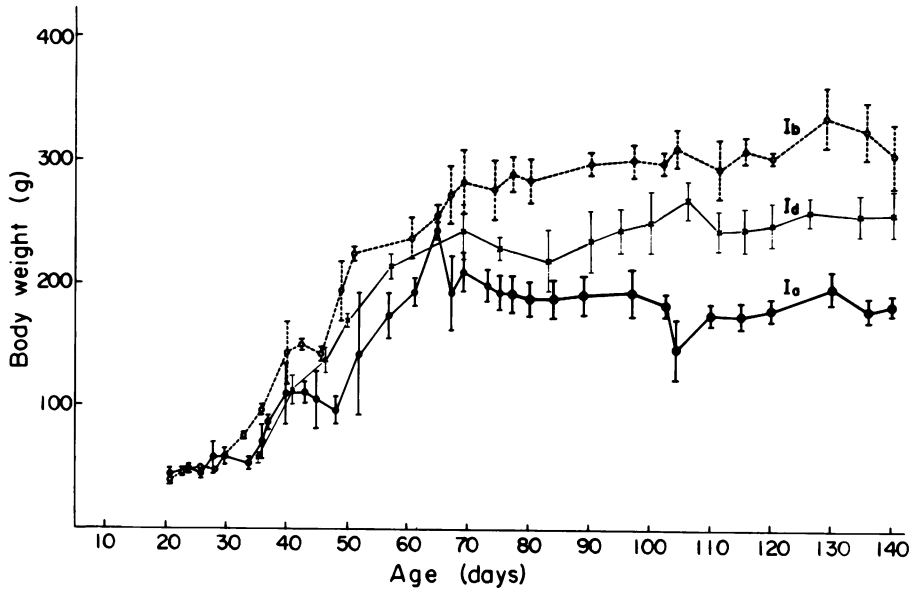
Serum insulin concentrations were measured by radioimmunoassay with Riakit (Dinabot Radioisotope Institute, Tokyo). Blood insulin responses to glucose loading were measured on rats fasted overnight and loaded orally with glucose (1.25 g/kg body weight).

Histologic and Histochemical Observation

Liver, pancreas, kidneys, spleen, adrenal glands, heart muscle, skin from the ear, and bone marrow were fixed with 10% formalin in phosphate-buffered saline (PBS), pH 7.0, for hematoxylin and eosin stain, Van Gieson stain, and Perl's Fe reaction. For zinc, the organs were fixed with pure ethanol solution; for pancreatic islet beta-granules, the organs were fixed in the Bouin solution. Islet beta-granules were stained by the modified method of Gomori; zinc was stained by the Okamoto method.[14]

Results

Group Ia rats showed a lower mean growth rate than the control groups, and after approximately 60 days of Fe^{3+}-NTA injection, body weight growth ceased at approximately 180 g (Text-figure 1). After 60 days of injection, these animals manifested hyperglycemia (173 ± 16 mg/dl [M ± SD] at Day 100), glycosuria (150 to 300 mg/dl at Day 100), ketonemia (40 mg/dl at Day 100), and ketonuria (10 mg/dl at Day 100), as measured by Ames Dexter Dexterstick. No glucosuria, ketonemia, or ketonuria was

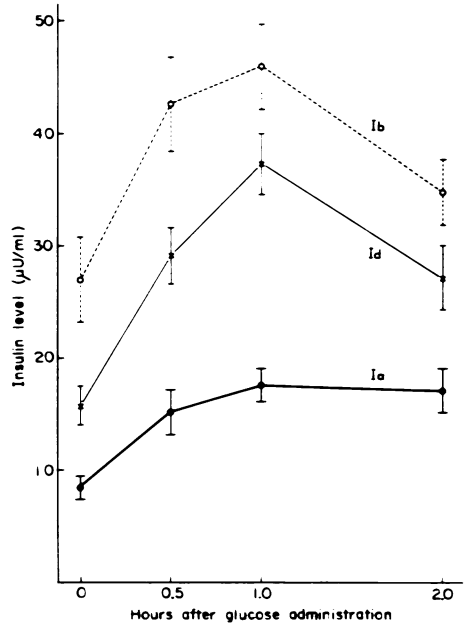


TEXT-FIGURE 1—Body weight (mean ± SD) of Fe^{3+}-NTA-injected rats (Group Ia), NTA-injected rats (Group Ib), and untreated control rats (Group Id). Injection began on Day 20 and continued until Day 140. There were 30 rats in each group.

present in Group Ib, Ic, or Id animals. Group Ia animals showed the typical diabetic response to overnight fasting; ie, both urinary and blood acetone concentrations were strikingly elevated (40 mg/dl and 80 to 100 mg/dl, respectively) while blood sugar levels dropped (84 ± 12 mg/dl) to near the control level (79 ± 10 mg/dl).

Oral glucose loading (1.2 g/kg body weight) was conducted on Day 100 of Fe^{3+}-NTA injection. Blood samples were taken before and at 0.5, 1.0, and 2.0 hours after glucose loading. The initial blood insulin concentration was significantly lower in Fe^{3+}-NTA-injected rats (Group Ia) than in control rats (Group Id) (Text-figure 2). The initial blood insulin level was remarkably higher in Group Ib than in Group Id. The insulin response to glucose loading was poor in Group Ia, compared with Group Id. The insulin response was higher in the NTA-injected rats (Group Ib) than in Group Id.

The liver and pancreas of Group Ia rats showed marked iron deposits. In liver sections prepared with Perl's stain, heavy granular iron deposits were found in parenchymal cells (Figure 1). In the pancreas, granular iron was found along the acinar lumen of exocrine cells, and it is noteworthy that some faint iron staining was present in islet cells located in the central area (Figure 3). Kupffer cells and pancreatic macrophages were moder-



TEXT-FIGURE 2—Blood insulin concentrations (mean ± SD) before and after oral glucose loading (1.2 g kg body weight). Group Ia, diabetic rats receiving Fe^{3+}-NTA injection (N = 10). Group Ib, nondiabetic rats receiving NTA injection (N = 10). Group Id, untreated controls (N = 7).

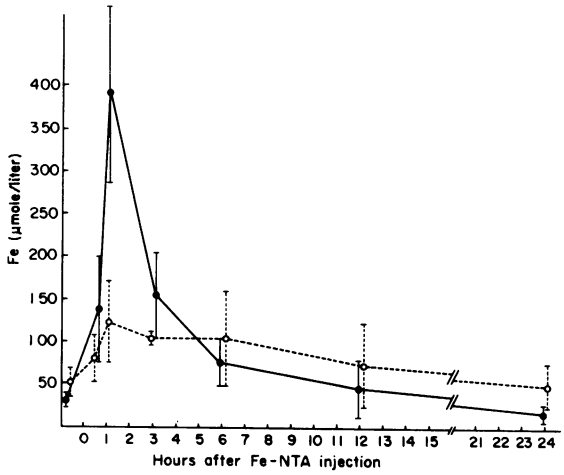
ately stained, but the cell swelling differed from that in animals phagocytizing colloidal iron. Iron deposition was also found in the adrenal medulla and sweat glands. However, kidney tubular cells and heart muscle cells remained free of iron.

In rats treated with colloidal iron (Group Ic), heavy iron deposits were found in the liver but mainly in Kupffer cells (Figure 2). Parenchymal cells showed weak staining. In the pancreas, iron was found only in macrophages of the connective tissues. Neither exocrine gland cells nor islet cells showed a positive iron reaction (Figure 4).

In pancreatic tissue of Group Ia animals stained by the modified Gomori method, a decrease was evident in the number of beta-granules. The polarization tendency of islet cell granules toward the vascular lumen was reduced. However, no evidence of heterogeneity in nuclear size or in cytoplasmic degeneration was observed (Figure 5).

In rabbits injected with Fe^{3+}-NTA, hyperglycemia, glycosuria, ketonemia, and ketonuria were also manifested within 3 months of iron injection; the tissue iron distribution was almost the same as in rats; and zinc staining in the islet cells was reduced (Figure 7), compared with that in the control (Figure 8).

In rats receiving a single intraperitoneal injection of Fe^{3+}-NTA (1.0 mg Fe/100 g body weight) (Group II), serum iron increased rapidly to a



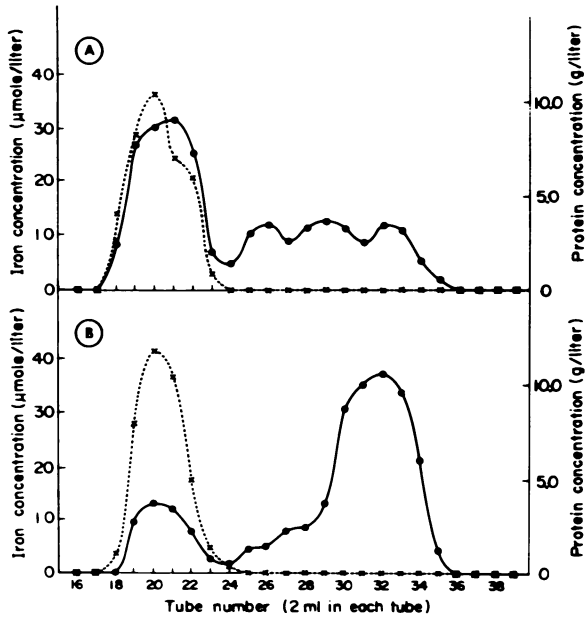
TEXT-FIGURE 3—TIBC and iron concentration (mean ± SD) in serum after a single intraperitoneal injection of Fe^{3+}-NTA (1.0 mg Fe/100 g body weight) in Group II rats. Each point represents the mean of 8 animals. The *vertical lines* represent standard deviations. *Dark circles*, iron concentrations in serum; *open circles*, total iron-binding capacity.

maximum at 1 hour after iron administration. At this time the serum iron concentration exceeded the total iron-binding capacity (TIBC) level but dropped sharply to below TIBC at 5 hours and returned to the original concentration within 24 hours (Text-figure 3). Serum samples taken at the maximum serum iron level eluted from a Sephadex G-15 column with 0.05 M Tris-HCl buffer, pH 7.4, indicating that most iron was bound to serum protein. A large front peak was followed by smaller free Fe^{3+}-NTA peaks. However, after elution with an equal volume of 0.05 M Tris-HCl and 0.05 M disodium-NTA, pH 7.4, the protein-bound iron peak nearly disappeared and a large second peak appeared, corresponding to the Fe^{3+}-NTA monomer (Text-figure 4).

Group III animals received a total of 43 mg of iron during 60 injection days. After four weekly phlebotomies (2 ml blood removed each week), there was a dramatic disappearance of hyperglycemia, glycosuria (Text-figure 5), ketonemia, and ketonuria. Histologic examination showed the absence of iron granules from liver parenchymal cells and pancreatic exocrine cells. The number of beta-cell granules was restored to normal.

Most animals in Group IV died of severe diabetes and pneumonia after 4 to 6 months of iron injection. Only 2 of 24 animals were living at the end of 6 months. The total amount of iron injected was 280 mg in each of these two survivors. Severe iron deposition in liver parenchymal cells was found in all animals, but no cirrhotic change was found in the liver and pancreas in any animal, including the 2 animals that survived for 6 months.

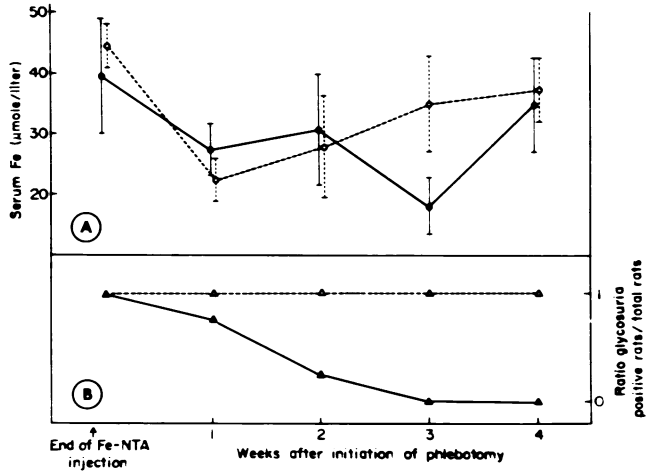
The stainable iron in the central cells of the pancreatic islets appeared slightly thicker in Group IV than in Group I. No necrotic change was detected in the islet cells which had clear nuclei.



TEXT-FIGURE 4—Chromatography of serum proteins and iron in Group II rats 60 minutes after a single intraperitoneal injection of Fe^{3+}-NTA. Serum was eluted through Sephadex G-15 in a 2.0 × 35 cm column. Elution buffer: **A**, Tris HCl, 0.05 M buffer, pH 7.4; **B**, Tris HCl, 0.025 M, containing 0.025 M free NTA, pH 7.4. *Dark circles*, iron concentration; *crosses*, protein concentration.

Discussion

Intraperitoneal injections of Fe^{3+}-NTA into rats and rabbits caused granular iron deposits in parenchymal cells of the liver, pancreas, and adrenal glands. Hyperglycemia, glycosuria, ketonemia, and ketonuria



TEXT-FIGURE 5—Effects of blood withdrawal on serum iron level (**A**) and glycosuria (**B**) in Group III rats. Rats were injected with 43 mg of iron over 2 months. After termination of iron administration, 2 ml of blood was withdrawn once weekly for 4 consecutive weeks. *Dark circles*, diabetic rats (N = 12); *open circles*, healthy controls (N = 12); *dark triangles*, diabetic rats with blood withdrawn (N = 12); *open triangles*, diabetic rats with no blood drawing (N = 12). Values are shown as mean ± SD.

were evident. These effects are similar to those in human hemochromatosis or bronze diabetes, except for the absence of liver cirrhosis and skin pigmentation. NTA itself was not toxic. Animals injected with only NTA in the same amount for the same period as those receiving Fe^{3+}-NTA (Group Ia) were in good nutrient condition, with a higher average body weight than the untreated controls.

The poor serum insulin response to oral glucose loading in the Fe^{3+}-NTA–treated animals suggests that the diabetic state was due to dysfunction of pancreatic islet cells. A decrease was found in beta-granules, although some recovery was observed after overnight fasting. The zinc reaction of islet cells was reduced in diabetic rabbits. The iron deposits may be related to islet dysfunction, although the amount of iron detected in beta-cells was small.

It has been generally believed that zinc in pancreatic islet cells is effective in stabilizing insulin.[22,23] The positive iron reaction of the pancreatic islet cells seems to suggest that zinc might be replaced by iron. However, a massive administration of Zn^{2+}-NTA to these Fe^{3+}-NTA–treated rats did not show a curative effect.[24]

Bates and his associates [15] demonstrated that iron bound to NTA is efficiently transferred to transferrin *in vitro* without forming spurious iron hydroxide complexes at neutral pH. It was also demonstrated that transferrin transferred iron to liver parenchymal cells rather than to Kupffer cells.[25]

After injection of large amounts of Fe^{3+}-NTA, transferrin was completely saturated immediately and the excess Fe^{3+}-NTA remained in serum, being associated with serum albumin. The association between Fe^{3+}-NTA and serum protein was loose and reversible, and such a complex may be responsible for inducing diabetes. Jacobs and his associates demonstrated that Fe^{3+}-NTA is taken up more efficiently by cultivated liver parenchymal cells than by transferrin iron *in vitro*.[26]

The association between Fe^{3+}-NTA and serum proteins probably explains the reduction of excretion of injected Fe^{3+}-NTA in urine and is probably related to heavy iron deposits.

One difference from human hemochromatosis was that no fibrotic changes were observed in the liver, pancreas, and other organs. This is consistent with observations of MacDonald and Pechet,[27] who suggested that cirrhosis in hemochromatosis may not be directly related to disturbances in iron metabolism but reflect other causes of tissue damage and incidental iron excess.

The therapeutic effect of repeated blood withdrawal indicated that the iron deposited in beta-cells is responsible for dysfunction of these cells.

Thus, our experiment directly suggests that disturbances in iron metabolism or excess iron deposition in pancreatic islet cells is the cause of diabetes in hemochromatosis. Although liver cirrhosis and skin pigmentation were not produced, this Fe^{3+}-NTA regimen appears to be useful for a variety of studies on hemochromatosis.

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Acknowledgments

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Legends for Figures

Figure 1—Liver section from Fe^{3+}-NTA-injected diabetic rat (Group Ia). Note heavy granular iron deposits in parenchymal cells and only moderate iron staining in Kupffer cells. (Perl's iron stain, ×400) **Figure 2**—Liver section from colloidal-iron-injected rat (Group Ic). Note heavy iron deposits in Kupffer cells and only weak staining in parenchymal cells. (Perl's iron stain, ×400) **Figure 3**—Pancreas section from Fe^{3+}-NTA-injected diabetic rat (Group Ia). Note granular iron deposition along the acinar lumen of exocrine cells and some faint iron staining in islet cells located in the central area. (Perl's iron stain, ×300) **Figure 4**—Pancreas section from colloidal-iron-injected rat (Group Ic). Note heavy iron deposits in macrophages of the connective tissues. There are no deposits in exocrine gland cells or islet cells. (Perl's iron stain, ×300) **Figure 5**—Pancreas section showing islet cells of Fe^{3+}-NTA-injected diabetic rat (Group Ia). Note decreased polarization of beta-cell granules toward the vascular lumen and a decrease in the number of beta-granules. (Aldehyde fuchsin Masson stain, ×300) **Figure 6**—Pancreas section showing islet cells of an untreated control rat (Group Id). Note the polarization tendency of beta-cell granules toward the vascular lumen. (Aldehyde fuchsin Masson stain, ×300) **Figure 7**—Pancreas section showing islet cells of a diabetic rabbit injected with Fe^{3+}-NTA. Note the reduction of zinc in the islet. (Zinc stain by the Okamoto method, ×400) **Figure 8**—Pancreas section showing islet cells of an untreated control rabbit. Zinc appears pinkish red. (Zinc stain by the Okamoto method, ×400)