Regarding 7-dehydrocholesterol, readily detectable in animals treated with AY-9944, the level in the serum reflects the rate of its biosynthesis(12) and degradation as well as its transportability by serum lipoproteins. Concerning the rate of biosynthesis, any dose of AY-9944 higher than the minimal dose which completely blocks cholesterol formation from 7-dehydrocholesterol should finally cause a similar, maximal concentration of serum 7-dehydrocholesterol. Actually, in pigs, higher doses and prolonged administration of AY-9944 resulted in progressively higher serum 7-dehydrocholesterol levels. Assuming the rate of catabolism of 7-dehydrocholesterol was not affected, this observation suggests enhanced biosynthesis of 7-dehydrocholesterol in pigs treated with AY-9944 as compared to that of cholesterol in untreated controls. The increase may indicate failure of the biosynthesized 7-dehydrocholesterol to activate the negative feedback mechanism of homeostatic control of cholesterogenesis normally triggered off by elevated levels of cholesterol(13).

The striking elevation of serum 7-dehydrocholesterol following prolonged administration of the highest dose to pigs may be indicative of secondary effects of AY-9944 on serum lipoproteins.

Summary. AY-9944, an orally active inhibitor of cholesterol biosynthesis, caused in rats, pigs and dogs greatly reduced serum cholesterol levels, only partly compensated by 7-dehydrocholesterol formed instead of cholesterol by the action of AY-9944. The distribution of 7-dehydrocholesterol between serum lipoprotein fractions differed from cholesterol and varied from species to species. Levels of 7-dehydrocholesterol and their distribution may reflect the rate of sterol biosynthesis.

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Regeneration of Insulin from its Inactive Reduction Product in the Presence of Pancreatic Islets. (31367)

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Tomizawa and Halsey(1) isolated an insulin degrading enzyme from beef liver. Tomizawa(2) and Katzen and Stetten(3) demonstrated that this liver enzyme catalyzes the reductive cleavage of the interchain disulfide bonds of insulin by virtue of H^+ transfer from reduced glutathione (GSH) and they proposed the name glutathione-insulin transhydrogenase for the enzyme.

Subsequently, Katzen, Tietze and Stetten

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(4) demonstrated that in the presence of oxidized glutathione, this enzyme favors the reconstitution of the disulfide bridges of insulin leading to the formation of biologically active hormone from its inactive reduction products.

Kotoulas, Morrison, and Recant(5) described a method for testing glutathione-insulin transhydrogenase activity in microquantities of tissue and demonstrated the presence of this activity in isolated pancreatic islets. The present work was undertaken to determine whether isolated pancreatic islets are capable of promoting the regeneration of insulin from its reduced and inactivated products.

Materials and methods. Crystalline beef zinc insulin, assaying 25.2 units/mg was a gift from the Eli Lilly Co. Oxidized glutathione was received from the Sigma Chemical Co., and 2-mercaptoethanol from Eastman Organic Chemicals, Rochester, N. Y.

A. *Preparation of the tissue.* Pancreatic islets and other tissues used were derived from normal Sprague-Dawley rats weighing 300 g and maintained on an *ad lib* Purina chow diet. Non-fasted rats were sacrificed by decapitation. The splenic portion of the pancrease was immediately excised and dropped into liquid nitrogen. Sections of the pancrease or other tissues were cut at 30 μ in a cryostat at —20°C and dehydrated *in vacuo* at —40°. The dried sections were stored up to 10 days at —70° in evacuated tubes. Dissection was carried out in a dehumidified room at 20° during a period not longer than 6½ hours. Islets and other rat tissues were dissected out by hand under a microscope at a magnification of 36×. The identity of the islets and other tissues was readily established on unstained sections and was confirmed on alternate stained sections. The periphery of each islet was cut away, leaving only the central portion for analysis to assure against contamination with non-islet tissue. The dry weights of dissected islet and other tissues on a quartz beam balance, ranged from 0.07 to 0.42 γ with a mean of 0.20 γ per islet. (Data obtained from 1200 weighed islets.) The islets and other tissues were then stored in microtubes, in a vacuum, at —70°, not longer than 15 days.

B. *Experimental procedure.* The technic used was similar to that employed by Katzen, Tietze and Stetten in their hepatic glutathione-insulin transhydrogenase studies(4), with special modifications and microadaptations in order to test the effect of tissues in microquantities. Insulin inactivation was accomplished by reduction in 2-mercaptoethanol. The regeneration of biologically(6) and immunologically(7) active insulin from the inactive reduction products was then measured. The effect of islet and other tissues was compared with the spontaneous reconstitution of disulfide bonds from the sulfhydryl groups of insulin.

The storage microtubes with the islets were gradually brought to room temperature while still under vacuum. Immediately after the vacuum tube was opened, small quantities of 0.1 M potassium phosphate buffer containing 5 mM EDTA and 0.2% gelatin were added to the microtubes (usually 30 lambda of buffer for 10-12 γ tissue). The microtubes were kept at room temperature for 30 minutes with frequent shaking (not buzzing). Then all microtubes were pooled and kept for another 15 minutes with frequent shaking. Small quantities of this islet-containing buffer were then transferred to the experimental microtubes where intact insulin in the same buffer was already present. (The experimental microtubes were diameter 6 mm, length 50 mm.) In addition 2-3 lambda aliquots were taken from the pooled islet solutions, placed in 50 lambda of buffer and tested for insulin content.

The resulting complete system in the experimental microtube had a total volume of 50 lambda, 100,000 μU of added insulin and 13.0-15.5 γ tissue (dry weight corresponding to about 65-78 islets). Control microtubes contained 1) insulin without tissue, 2) tissue without insulin, and 3) 16 γ human albumin with insulin. Then 1.59 lambda of 2-mercaptoethanol was added to each microtube. The tubes remained at room temperature over a 60-minute period with occasional mild shaking. The addition of mercaptoethanol leads to complete inactivation of the insulin under the above conditions. At the end of this period, the contents of each microtube were

TABLE I. Insulin Content of Microdissected Rat Islets.*

Animal No.	Method	
	Fat pad assay	Immunoassay
1	678	
2		520
3	204	
4	135	
5	945	
6	765	
Mean	545	

* Values expressed as μunits/γ dry weight.

dialyzed by closure of the open end of the tube with a single wall of a dialysis sac (Fisher) inversion of the tube and immersion, about 1 cm deep, in a beaker containing 35 ml of the phosphate-EDTA-gelatin buffer. The dialysis was continued for 1, 2, or 4 hours at 25°C, with magnetic stirring and complete buffer changes every 10 minutes. With every change in buffer the microtube was inverted. This permitted washing down of the entire inside wall of the microtube. At the end of the first hour there was still a slight odor of mercaptoethanol in the microtube.

To determine the insulin content, microtubes were withdrawn at various times after dialysis, incubated for 45 minutes at 37°C under nitrogen, kept for 12 hours at 4°C, and then the volume was brought to 7 ml by addition of Krebs-Ringer bicarbonate buffer (pH 7.4). Insulin was assayed then by the rat fat pad assay and/or by the immunoassay. The effect of oxidized glutathione (GSSG) upon the regeneration of insulin was determined by adding GSSG to the buffer against which the microtubes were dialyzed (110 mg GSSG/100 ml buffer). In every microtube subjected to dialysis with GSSG, before the incubation at 37°C 5 lambda of a solution of GSSG (200 mg/ml) was added.

Results. Table I shows the mean insulin content of dissected islets to be 545 μU per γ of dry weight. The initial insulin content of the microtube represented the added insulin (100,000 μU) plus the amount of insulin due to the islets. The latter was determined from an aliquot of pooled islets used in each experiment and measured by direct insulin assay. The added insulin due to islets ranged from 2,100 μU to 14,644 μU/microtube, comprising not more than 14% of the insulin content of the microtube.

Table II shows the changes of insulin content in the microtubes during dialysis, expressed as % of the initial insulin content. It appears that in the presence of islet tissue, there is significant insulin regeneration by the end of the second hour, while only very small amounts of active insulin exist at the end of the first hour of dialysis. Control microtubes lacking tissue showed less than 0.20% of the initial insulin content of the complete system when assayed by the fat pad method. The same controls tested by immunoassay also revealed very low insulin contents not exceeding 0.25% of the initial insulin content of the complete system. Control microtubes lacking added insulin but containing islets showed no measurable activity by fat pad assay.

Oxidized glutathione was added to the dialyzing fluid at the beginning of the second and third hour of dialysis in order to determine if this compound could serve as a hydrogen acceptor, promoting the regeneration of active insulin. After addition of oxidized glutathione at the beginning of the second hour, no enhanced regeneration of insulin could be demonstrated. The addition of the

TABLE II. Effect of Pancreatic Islets on Regeneration of Reduced Insulin.

Insulin plus islets	Method of insulin assay	% Initial insulin at Hr of dialysis 1	2	4
1	Fat pad	>.21	1.81	
	Immuno	.07	.63	
2	Fat pad	>.21	.94	
3	Immuno	.00	1.31	
4	Fat pad			1.04
	Immuno			1.47
Insulin without islets				
1	Fat pad	.10	<.05	.08
2	" "	<.04	<.04	
3	Immuno	.12	.25	.17
4	Fat pad			<.21
	Immuno			.19

Measurements were made after reduction of insulin with 2-mercaptoethanol and following dialysis against 0.1 M potassium phosphate, 5 mM EDTA, 0.2% gelatin buffer.

TABLE III. Effect of Oxidized Glutathione on Insulin Regeneration.*

Insulin + islets	% Initial insulin as measured by	
No. of exp	Fat pad assay	Immunoassay
1	.53	.65
2	.23	.10
3	.20	
4	.54	
5	.25	

* After reduction of insulin and 2 hr of dialysis as in Table II, a further 2 hr dialysis against glutathione was carried out.

compound at the beginning of the third hour of dialysis led to no increase but rather to a decrease in insulin content (Table III).

To determine whether the enhanced regeneration of insulin observed in the presence of the islets was a non-specific binding effect associated with protein, human albumin was substituted for the islets. These studies showed 0.21% insulin content by immunoassay. Preliminary studies with other tissues of the rat revealed the presence of insulin regenerating activity in many tissues (Table IV).

Discussion. It should be noted that the paucity of observations is due to the enormous number of microdissected islets required for each experiment. Despite this it appears that pancreatic islets are capable of promoting the reconstitution of the disulfide bonds from the sulfhydryl groups of insulin, leading to the formation of biologically and immunologically active hormone from its inactive reduction products. This reaction may involve an electron acceptor present in the tissue used. It is of interest that the pancreatic acinar tissue may be even more active in this regard, and that apparently other tissues also can function in this manner.

TABLE IV. Effect of Various Tissues on Insulin Regeneration.*

Tissue	% Initial insulin added
Muscle of diaphragm	.61
Kidney (cortex)	.47
Brain (grey and white matter, equally)	.54
Striated muscle	.38
Pancreas (acinar tissue)	2.45
Intestine (mucosa & muscularis layers)	.07
Spleen	.47
Myocardium	.75
Albumin	.21
No tissue	.19

* Insulin content of the dialysis microtube after reduction with 2-mercaptoethanol and during a 4-hr dialysis against 0.1 M potassium phosphate—5 mM EDTA—0.2% gelatin buffer in presence of 13-15 γ dry weight various tissues. Insulin assayed by immunoassay.

The adverse effect of oxidized glutathione on this system cannot be explained by these data. It is possible that the oxidation of sulfhydryl groups present in the system by the oxidized glutathione leads to activation of tissue processes resulting in destruction of regenerated insulin.

The mechanism involved in the tissue enhanced regeneration is not clear. It does not seem to be associated with increased binding of insulin and prevention of some loss through the dialysis membrane, since albumin could not substitute for tissue. Though there appears to be a difference between tissues, the observations are too few to assess these differences as absolute.

Perhaps the most interesting possibilities relate to the potential of this regeneration of insulin from reduced chains as a fundamental mechanism in the synthesis of insulin in islets. The observations of Humbel(8) indicate the likelihood that insulin is synthesized as 2 separate chains. Whether the role of other tissues in this enhanced regeneration is significant to the physiologic mechanism of insulin action can only be hypothesized.

Summary. A microtechnic for testing the regeneration of biologically and immunologically active insulin from its inactive reduction products is described. Pancreatic islets as well as certain other tissues appear to have a biological activity which promotes this regeneration.

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Identification of LSc2ab Strain of Type 1 Poliovirus by Inhibition With Horse Serum and Dextran Sulfate.* (31368)

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Means are now available to distinguish the LSc2ab attenuated virus from a limited number of other type 1 strains of poliovirus provided that their characteristics are known. However, genetically determined markers of the LSc2ab virus (Sabin type 1 vaccine) that will distinguish it from all other type 1 strains, especially wild strains, are still being sought. Most characteristics of LSc2ab are either not specific (*e.g.*, replicative capacity at 40°C) or are too unstable on passage of the virus to differentiate it with certainty (*e.g.*, intratypic immunologic properties). In order to provide a reliable means of distinguishing the LSc2ab virus and its subpassages from other type 1 viruses, whether attenuated or virulent, we have tested the use of two different antiviral substances in combination(1-2). Each inhibitor has a greater effect on LSc2ab than on other type 1 strains; both inhibitors combined have an additive anti-LSc2ab effect. Moreover, mutants resistant to one inhibitor (equine serum) are rare, while mutants resistant to the other inhibitor (dextran sulfate), although rather frequent, appear to retain their sensitivity to the equine serum.

Materials and methods. Cell cultures and media. Primary Rhesus monkey kidney (MK) cells were grown in 60 mm plastic petri dishes with Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS) non-inhibitory for poliovirus, penicillin (100 I.U./ml), streptomycin (100 μg/ml) and nystatin (20 μg/ml) in a humidified atmosphere of 5% CO_2 and air.

Viruses. All strains were type 1. Pools of the attenuated CHAT and LSc2ab vaccines were stored in small aliquots at −20°C for use without further passage. Mahoney virus was prepared by infecting MK cells at an input multiplicity of 3 - 10 plaque-forming units (PFU); the virus-containing medium (MEM) was havested after 24 hours, centrifuged at 2000 rpm and the supernatant kept at −20°C.

The wild type 1 strains, previously described(3), were isolated in different parts of the United States and Puerto Rico from asymptomatic persons and patients with poliomyelitis and had been passed 3 to 4 times in MK cells.† The strains received a further mass passage but were never plaque-passed.

First human-passage strains of the CHAT and LSc2ab vaccines were obtained by vaccination of infants in isolated bassinets. Ten per cent suspensions of feces treated with antibiotics and centrifuged at a low speed were regarded as undiluted human-passage material. After dilution of the supernatants their virus content and characteristics in plaque reduction tests were examined directly without an intermediate passage in cell culture.

Plaque Assay. Duplicate monolayers of MK cells were inoculated with 0.2 ml of virus and placed at 37°C for 60 minutes. The cell cultures were then overlaid with 5 ml of medium consisting of liquefied 1% Noble's agar, 0.055% bovine albumin, 2.5% inactivated FCS, penicillin, streptomycin and nystatin. After 49 hours at 37°C a second overlay with 0.01% neutral red was added,

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† Strains courtesy of Dr. James Nakano.