process of transport of glucose in the intestine is, therefore, different from the transport mechanisms operating in muscles and is independent of the direct action of insulin.

Summary. Effect of insulin insufficiency on the transport of glucose in the intestinal cells was studied by incubation of small intestines of rats and guinea pigs in a medium containing glucose. Insulin deficiency was produced by injection of alloxan to rats and by feeding a scorbutogenic diet to guinea pigs. Concentrations of glucose in the intracellular water of the intestine incubated in glucose-saline were found higher in the intestines obtained from diabetic rats and from scorbutic guinea pigs as compared to normal. Insulin does not seem to have any direct role in the intracellular transport of glucose in the small intestine.

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Erythrocyte Hemolysis by Isolated Rat Liver Lysosomes.* (29887)

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Present knowledge of lysosomes has been summarized recently(1). Interaction between lysosomes and other membranous parts of cells is one of the most important yet least understood reactions. Purified lysosomes recently have become available and their composition and reactions of their lipoprotein membrane have been studied(2,3). This paper reports studies of *in vitro* hemolysis of erythrocytes by isolated rat liver lysosomes.

Experimental. Male Sprague-Dawley rats weighing about 200 g were used for collecting blood for erythrocytes and livers for isolating subcellular fractions—mitochondria, microsomes and lysosomes. Blood was collected by decapitating the rats and was immediately mixed with equal volume of ice-cold saline phosphate buffer pH 7.4 (0.9% saline in 0.1 M phosphate buffer). Livers also were removed immediately and were chilled in ice, washed and homogenized in a Waring blendor for 20 seconds in 0.25 M

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sucrose media containing 1 mM ethylenediamine tetraacetate (EDTA) to give a 10% liver homogenate preparation. The pH of this homogenate was adjusted to 7.0 by addition of NaOH and subjected to centrifugal fractionation procedure as follows:

Mitochondria. Supernatant obtained after removal of debris at 750 × *g* for 10 minutes was centrifuged at 500 × *g* for 10 minutes to obtain mitochondria. The pellets were washed once with saline-phosphate buffer pH 7.4 before making the final mitochondrial suspension.

Lysosomes. Supernatant after removal of mitochondria was subjected to sucrose-gradient centrifugation as described by Tappel *et al*(3) to obtain purified lysosomes. Lysosomal preparation was frozen and thawed for complete lysis and the final suspension was made in saline-phosphate buffer pH 7.4.

Microsomes. Supernatant remaining after removal of lysosomal fraction was centrifuged at 105,000 × *g* for 60 minutes to obtain microsomes. The pellets were washed once and suspended in saline-phosphate buffer pH 7.4.

Lysosome-subfractions. Lysosomal suspension after several freezing and thawing treatments was centrifuged at 100,000 × *g* for 2 hours to separate membrane from soluble fraction. Membrane fraction was then suspended in saline-phosphate buffer pH 7.4 to bring to the original volume of lysosomal suspension.

Erythrocytes. Ice-cold blood in saline-phosphate buffer was centrifuged at 750 × *g* for 10 minutes. The isolated erythrocyte cells were washed twice in saline-phosphate buffer and resuspended in 4 times its volume of ice-cold saline-phosphate buffer pH 7.4. This will be referred to as erythrocyte suspension throughout this study.

Hemolysis measurements. 0.25 ml of erythrocyte suspension was incubated with equal volume of either mitochondrial, microsomal or lysosomal suspension at 37°C for 4 hours. At the end of this period the reaction mixture was diluted to 5 ml volume by adding ice-cold saline-phosphate buffer pH 7.4. The clear supernatant was then obtained by centrifuging at 750 × *g* for 10 minutes. Hemolysis was determined by measuring released hemoglobin in the supernatant at 500 mμ. Results are expressed as % of total hemolysis obtained by incubating equivalent amount of erythrocyte suspension in de-ionized water, other experimental conditions remaining identical. Appropriate blanks and controls were run in each experiment and all results were appropriately corrected.

TABLE I. Effect of Different Subcellular Fractions on Erythrocyte Hemolysis.

Subcellular fractions*	% of total hemolysis†
Nil	0
Mitochondria	3
Microsomes	3
Lysosomes	20

* Protein equivalence 1 mg/ml.
† Equal volume of erythrocyte and lysosome suspensions incubated at 37°C for 4 hr. Values represent averages of 3 independent trials.

TABLE II. Erythrocyte Hemolysis at Different Levels of Added Lysosomes.

Lysosome concentration mg protein/ml	% of total hemolysis*
.0	0
1.0	32
1.6	58
2.0	61

* Equal volume of erythrocyte and lysosome suspension incubated at 37°C for 4 hr. Values represent averages of 3 independent trials.

Results. The effect of various rat liver fractions on the hemolysis of erythrocytes was determined. Results presented in Table I show that on an equivalent protein basis mitochondria and microsomes caused only 3% hemolysis as compared to 20% for lysosomes. This ability to hemolyze erythrocytes was nearly proportional to the lysosomal concentration (Table II).

Hemolysis of erythrocytes as a function of reaction time is presented in Fig. 1. The hemolysis reached a maximum of about 80% at the end of 5 hours.

Results in Table III show some typical characteristics of the hemolytic activity and its distribution in various subfractions of the isolated lysosomes. Of the total activity in lysosomes, 74% was found associated with the membrane and only 26% with the soluble or the non-membrane fraction. Heat treating

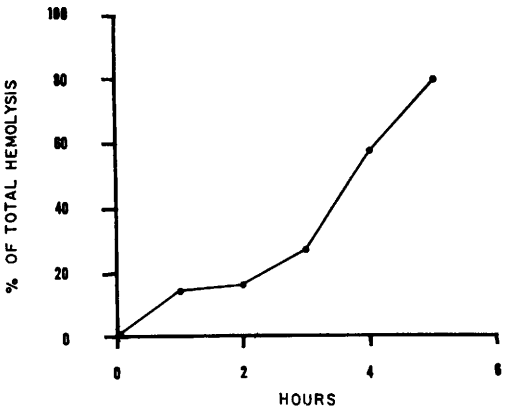

FIG. 1. Kinetics of erythrocyte hemolysis by lysosomes. Lysosome concentration: 1.5 mg lysosomal protein/ml of erythrocyte suspension. Incubation at 37°C for 4 hr. The figure represents a net difference between lysosomes plus erythrocyte and erythrocyte alone.

the lysosomes reduced hemolysis to 51% of the original, indicating that the hemolytic activity associated with lysosomes is partly enzymic and partly non-enzymic in nature. By removing the lipid components from lysosomes, employing repeated (4) extractions with a mixture of methanol and petroleum ether (1:2), the hemolytic activity of the lysosomes was reduced to half its original activity. The remaining activity apparently is associated with the lipid fraction of the lysosomes.

Results in Table IV show the effect of 3 metal coordination compounds, a lipid antioxidant and vit A on the erythrocyte hemolysis by lysosomes. All except vit A acid reduced the hemolytic effect of the lysosomes presumably by protecting the erythrocyte membrane. Vit A is known to activate erythrocyte hemolysis by its action on lipoprotein membrane(4).

TABLE III. Characteristics of Hemolytic Activity in Lysosomes.

Lysosomes and lysosomal fractions*	% of total hemolysis†
Unfractionated lysosomes	26.0
Membrane	19.2
Soluble	6.8
Heat treated lysosomes‡	13.3
Lipid extracted lysosomes	13.0

* Concentration of lysosomes was 1 mg protein/ml suspension and of lysosomal fractions was equivalent to their contents in unfractionated lysosomes.

† Equal volume of erythorcyte and lysosomal suspensions incubated at 37°C for 4 hours. Values represent averages of 3 independent trials.

‡ Obtained by heating in boiling water bath for 20 min.

Discussion. Since erythrocytes possess a lipoprotein type membrane, it is of considerable interest to study the action of isolated lysosomes on erythrocytes, using hemolysis as a criterion of membrane damage. Results shown in Table I demonstrate a definite hemolytic activity specific to lysosomes, since similar subcellular fractions such as mitochondria and microsomes did not hemolyze the erythrocytes to any appreciable extent. Furthermore, the hemolytic process was proportional to the concentration of lysosomes in the reaction system (Table II). Studies shown in Fig. 1 also indicate that the erythrocyte hemolysis in the presence of lysosomes progresses as a function of time, and at a rapid rate during the later period of incubation.

TABLE IV. Effect of Membrane-Active Compounds on Erythrocyte Hemolysis by Lysosomes.

Compound added 10^{-4} M	% of total hemolysis*
Lysosomes only	20
" + EDTA	17
" + succinate	15
" + citric acid	13
" + dl-α-tocopherol	9
" + vitamin A acid (Na salt)	82

* Equal volume of erythrocyte and lysosome suspensions (1 mg protein/ml) incubated at 37°C for 4 hr. Values represent averages of 3 independent trials.

After establishing the *in vitro* capacity of the lysosomes for hemolyzing erythrocytes, the next problem was to define some of the characteristics of the hemolytic agents. It appears from the results shown in Table III that three-fourths of the lysing activity is associated with the membrane components and that its distribution is equal between lipid and non-lipid fractions of the lysosomes. It is interesting to observe that heat inactivation of the lysosomal enzymes had significant effect in reducing the hemolytic activity but did not destroy it completely, thereby indicating that about half of the hemolysis of the erythrocytes under the present experimental conditions was contributed by the

action of hydrolytic enzymes and the rest by some unknown non-enzymic components capable of causing some lysing.

Certain levels of inhibition by 3 metal coordination compounds and the antioxidant (Table IV), although of not very large magnitude under the present experimental conditions, suggests that metal-catalyzed lipid peroxidation could also be a possible reaction leading to hemolysis. Tsen and Collier(5) showed that lipid peroxidation can be a dominant reaction leading to hemolysis of erythrocytes. Tissue fractions high in iron give increased catalysis of lipid peroxidation(6). Liver lysosomes are known to contain relatively large amounts of ferritin. Analysis of some of our preparations of lysosomes showed that they contained 2 mg iron, .1 mg copper and .03 mg manganese per gram protein.

The evidence obtained here also suggests that other hemolytic agents of lipoprotein nature contribute to the hemolytic activity of the lyososomes. Charge interactions or binding between the reactive groups on the membranes of both lysosomes and the erythrocytes may play an important role in the lysing process. Recently, a similar hemolytic factor has been extracted from necrotic portions of the hamster tumor(7) and had characteristics similar to those observed in the present study. It is apparent that, under the experimental conditions described, the factors contributing to hemolysis can possibly be ascribed to the following: lysosomal hydrolytic enzymes, lysosomal lipoprotein membrane, and lysosome heavy metals catalyzing lipid peroxidation. Further investigation into this complex of reactions requires further knowledge of the chemical composition and the structure of lysosomes.

Summary. Study has been made of the hemolytic activity of the isolated rat liver lysosomes. This activity, specific to lysosomes, was in proportion to their concentration in the reaction system. Under the experimental conditions described the hemolysis could be related to: lysosomal hydrolytic enzymes, the lysosome lipoprotein membrane and other factors such as lysosome heavy metals which may catalyze lipid peroxidation.

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Effects of 3,5-Dimethylisoxazole (U-21221) on Fat Metabolism. (29888)

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It was previously demonstrated that 3,5-dimethylisoxazole produced a marked effect on glucose metabolism(1). Since insulin is capable of depressing plasma free fatty acid (2) and since FFA may inhibit glucose utilization(3,4), it was of interest to study the effects of U-21221 on FFA. This paper describes the action of U-21221 on FFA metabolism and the possible relationship of this effect to observed changes in glucose metabolism.

Methods. Plasma FFA and blood sugar. Intact rats weighing 140-150 g were used following overnight fast. Animals used in evisceration studies weighed 180-200 g and were fasted overnight. Eviscerations were done as previously described, using spinal transectomized rats(1). In some studies on intact