

INFLUENCE OF GLUCAGON, AN INDUCER OF CELLULAR AUTOPHAGY, ON SOME PHYSICAL PROPERTIES OF RAT LIVER LYSOSOMES

RUSSELL L. DETER and CHRISTIAN DE DUVE

From The Rockefeller University, New York

ABSTRACT

The response of rat liver lysosomes to an intraperitoneal injection of glucagon has been evaluated from studies on the mechanical fragility, osmotic sensitivity, and sedimentation properties of these subcellular particles. It has been found that about $\frac{1}{2}$ hr after the injection of glucagon the hepatic lysosomes exhibit a fairly sudden increase in their sensitivity to mechanical stresses and to exposure to a decreased osmotic pressure. At the same time, their sedimentation properties undergo complex changes characterized mainly by a significant increase in the sedimentation coefficient of a considerable proportion of the total particles. In addition, glucagon causes an increase in the proportion of slowly sedimenting particles, with the result that the distribution of sedimentation coefficients within the total population tends to become bimodal. The latter change is more pronounced for acid phosphatase, less so for cathepsin D, and barely detectable for acid deoxyribonuclease. All these modifications are maximal between 45 and 90 min after injection and regress to normal within approximately 4 hr. With the exception of the increase in the slow component, for which no explanation can be advanced at the present time, they are consistent with the hypothesis that glucagon causes an increase in lysosomal size, and may be related to the autophagic-vacuole formation known to occur after glucagon administration.

INTRODUCTION

First noted by Clark (8) in newborn kidney, the phenomenon of cellular autophagy has now been observed in a variety of biological materials and in numerous experimental circumstances. Present indications are that it constitutes a basic cellular process of both physiological and pathological importance (for a review, see reference 11).

Except for a number of cytochemical staining data showing that autophagic vacuoles contain acid phosphatase and, presumably, other acid hydrolases, very little is known concerning the mechanisms whereby these structures are formed and their contents digested. The discovery by Ashford and Porter (2) that glucagon induces

autophagic vacuole formation in the perfused rat liver has opened the way toward a combined biochemical and morphological study of this phenomenon, by providing a means of evoking cellular autophagy reproducibly and under accurately timed conditions in a system in which lysosomes have been particularly well characterized. The present paper describes the results of a biochemical investigation of the changes undergone by hepatic lysosomes as a result of the injection of glucagon. The hormone was given to intact animals since Baudhuin has found that it is also effective under these conditions; the formation of numerous autophagic vacuoles, associated

with an apparent loss of pericanalicular dense bodies, is observed in the liver within an hour after injection (see reference 11). It has already been shown by Berthet (6) that this phenomenon is associated with evidence of increased fragility of hepatic lysosomes.

METHODS

All investigations were carried out on litters of two to four adult male Sprague-Dawley rats fasted for 7–8 hr. Crystalline glucagon (Eli Lilly & Co., Indianapolis) was injected intraperitoneally in about 0.2 ml of 0.2 M glycine-NaOH buffer pH 9.5; the same volume of buffer was given to one of the littermates which served as control. In early studies, the animals received 100 μ g of glucagon, regardless of weight, giving an average dose of 45.3 ± 8.5 μ g per 100 g body weight. In most experiments, the dose was 50 μ g per 100 g body weight. There is no indication from the experimental data that dosage was critical within the limits mentioned.

The animals were killed by decapitation at times ranging between 0 and 6 hr after injection; the livers were removed rapidly, placed in a tared beaker containing ice-cold 0.25 M sucrose, and weighed. Homogenates were prepared either with a Potter-Elvehjem (14) type homogenizer (A. H. Thomas Co., Philadelphia), made up of a smooth-walled glass tube

fitted with a cylindrical Teflon pestle driven by a motor at about 1000 rpm., or with an all-glass homogenizer (Kontes Glass Company, Vineland, New Jersey) of the type described by Dounce et al. (12) operated by hand. These two preparations will be referred to as *Potter homogenates* and *Dounce homogenates*, respectively. They were made up to a final volume of 10 ml per g fresh liver. In a number of experiments, the livers were disrupted by a method combining homogenization with separation into a *nuclear fraction* and a *cytoplasmic extract* (10). The details of this procedure, which is designed to minimize damage to subcellular particles, are shown in the upper part of Fig. 1. The nuclear fraction was made up to 5 ml per g of original fresh liver and the cytoplasmic extract to 10 ml per g liver. All manipulations were carefully standardized and every effort was made to avoid differences in the preparative procedure.

The degree of integrity of the lysosomes was evaluated by measurements of free acid phosphatase activity on whole homogenates or extracts, and by studies of the partition of acid hydrolases between supernatant and particulate fractions separated by centrifugation for 45 min at 100,000 *g* in the No. 40 rotor of the Spinco Model L ultracentrifuge. The mechanical fragility of the lysosomes was investigated on preparations subjected to additional up-and-down runs in the Potter homogenizer. The procedure of

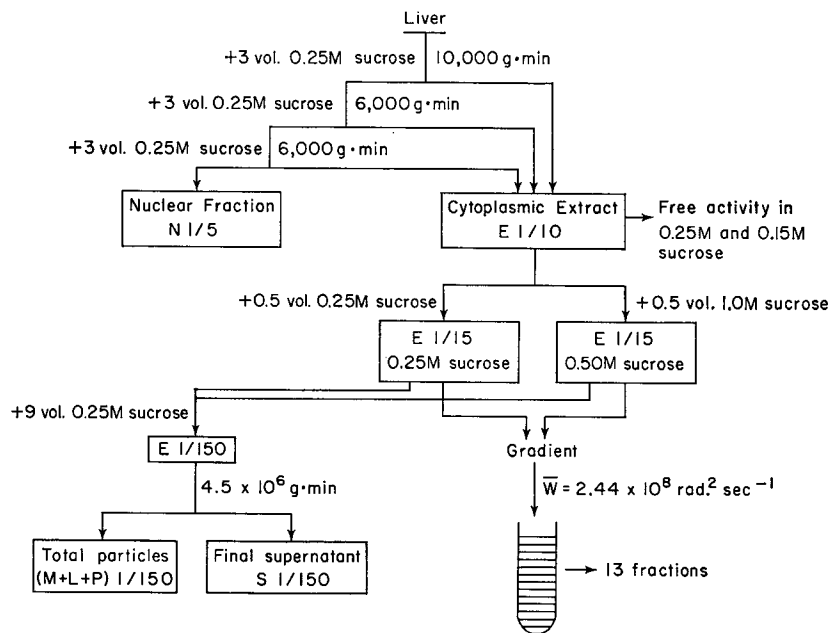


FIGURE 1 Summary of procedures used in sedimentation-velocity experiments. *N*, *E*, and *S* refer to nuclear fraction, cytoplasmic extract, and high-speed supernatant, respectively; *M* + *L* + *P* represents sum of cytoplasmic particles. Symbols are those used by de Duve et al. (10).

Appelmans and de Duve (1) was followed to assess their osmotic sensitivity. The preparations were diluted to final sucrose concentrations ranging between 0.20 and 0.025 M and kept at 0° for 30 min; they were then brought back to the original 0.25 M concentration by addition of a suitable amount of concentrated sucrose.

Sedimentation-velocity analysis was carried out on cytoplasmic extracts by differential density-gradient centrifugation, as described by Beaufay et al. (3). Tubes were filled with cytoplasmic extract at a constant concentration of 67 mg of fresh liver per ml, stabilized by a linear sucrose gradient ranging from 0.25 to 0.5 M (density range: 1.034–1.067). Preparations from a glucagon-treated animal and a littermate control were centrifuged simultaneously in the SW-39 swinging bucket rotor in the Spinco Model L preparative ultracentrifuge under conditions designed to cause incomplete sedimentation of the particles. Continuous recording of the rotational speed made it possible to express the centrifugation conditions accurately as W , the time-integral of the squared angular velocity (9). On an average, W was equal to $2.44 \times 10^8 \text{ rad}^2 \text{ sec}^{-1}$. After centrifugation, the tubes were sectioned to provide 13 fractions which were collected in tared tubes filled with a known volume of a solution containing mM sodium bicarbonate, mM ethylenediaminetetra-acetic acid and 0.01% of Triton X-100. All operations were performed according to Beaufay et al. (3, 4) with the equipment described by de Duve

et al. (9) and at a temperature of 0–2°. Special care was taken to reduce convection artifacts by following the precautions recommended by these authors. A portion of the extracts used in preparing the gradients was also centrifuged at high speed for the determination of unsedimentable activities, as described above. All the fractions as well as the original extract were assayed for various enzymes. The extracts were also analyzed for free acid phosphatase activity before and after exposure to 0.15 M sucrose, to ascertain the state of the lysosomes and their sensitivity to an osmotic shock. The complete design followed in this group of experiments is summarized in Fig. 1.

Assays for cytochrome oxidase, acid phosphatase, cathepsin D, and acid deoxyribonuclease were made essentially as described by Beaufay et al. (3). A kinetic study of the deoxyribonuclease reaction revealed that the liberation of acid-soluble nucleotides follows a slightly sigmoid curve; for greater accuracy, the results obtained for this enzyme were evaluated graphically by means of a standard curve. Free acid phosphatase was measured according to Gianetto and de Duve (13). The method of de Duve et al. (10) was used for the assay of glucose-6-phosphatase.

The results of the enzyme determinations are given either in absolute units, as defined by de Duve et al. (10), or as percentage of the activity of the starting material to provide an estimate of the recovery. The results of the gradient experiments were calculated and plotted in histogram form essentially as de-

TABLE I
Influence of Glucagon on Total Enzyme Activities

Values given are means \pm standard error. Values in the last column are averages \pm standard error of differences between pairs of littermates. The number of individual values in each group is given in parentheses.

Enzyme	Time after glucagon	Units* per g liver (wet weight)		
		Control	Glucagon	Difference (G-C) between littermates
	<i>min</i>			
Acid phosphatase	0	8.74 \pm 0.55(4)	8.16 \pm 0.28(10)	
	45	9.15 \pm 0.47(13)	9.28 \pm 0.58(15)	+0.16 \pm 0.47(9)
	90	9.78 \pm 1.06(23)	9.25 \pm 1.08(26)	-0.18 \pm 0.28(20)
	120	8.84 \pm 0.23(8)	8.47 \pm 0.20(13)	-0.10 \pm 0.33(8)
				Combined
				-0.08 \pm 0.20(37)
Cathepsin D	45	0.81 \pm 0.36(6)	0.90 \pm 0.16(6)	+0.09 \pm 0.04(6)
Acid deoxyribo-nuclease	90	1.51 \pm 0.16(6)	1.45 \pm 0.17(6)	-0.06 \pm 0.09(6)

* One unit is the amount of enzyme releasing one μ mole of inorganic phosphate (acid phosphatase), of tyrosine equivalents (cathepsin D) or of mononucleotide equivalents (acid deoxyribonuclease), per minute under the conditions of the assays (10).

scribed by Beaufay et al. (4). They were further converted to a sedimentation coefficient scale by means of the approximate formula given by Beaufay et al. (3), assuming a simple linear relationship between sedimentation coefficient and radial distance. When several histograms were combined, their abscissa scales were divided into 12 equal sections and the fractional areas covered by the same section in each histogram were computed and averaged. A new histogram was constructed with the averaged values. Most of these calculations were made by means of a Control Data Corporation 160-A digital computer.

The treatise by Steel and Torrie (15) was used as a guide in the statistical calculations. Means are given with their standard error. When the experimental

design permitted it, the results were analyzed by factorial variance analysis and the significance of the observed effects was estimated from the value of the variance ratio *F*. Differences between individual means were evaluated by the multiple range test of Student-Newman-Keuls.

RESULTS

Influence of Glucagon on Total Enzymatic Activities

In Table I are listed the total acid phosphatase, cathepsin D, and acid deoxyribonuclease activities recorded in our longest series of deter-

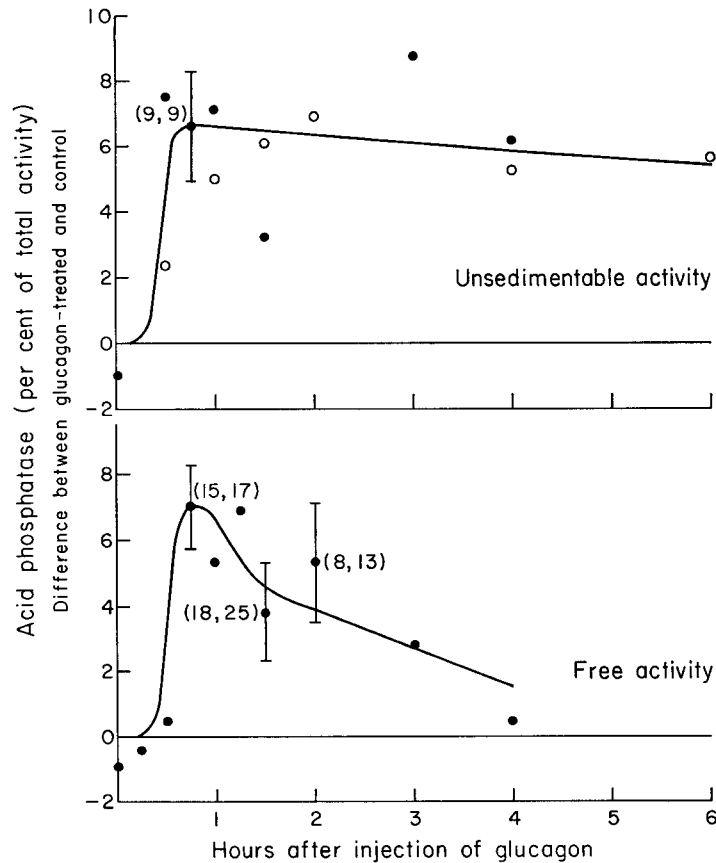


FIGURE 2 Effect of glucagon on free and total acid phosphatase activities. Results plotted are averages of differences observed between glucagon-treated animals and littermate controls, killed at the same time after injection. Larger series are shown with their standard error of mean and, in parentheses, the number of control and glucagon-injected animals. Other points refer to single animals or to smaller series. In graph of unsedimentable activities, solid circles refer to cytoplasmic extracts, open circles to homogenates. Free activity values obtained on homogenates and on cytoplasmic extracts did not differ significantly and were pooled. All preparations were made with the same Potter homogenizer. Free activity values from glucagon-treated animals represent a total of 89 determinations as compared to 58 in controls; the total number of unsedimentable activity values are 33 and 21, respectively.

TABLE II

Free and Unsedimentable Acid Phosphatase Activities of Controls

Values are pooled from animals killed at times varying between 0 and 6 hr after injection of solvent. All preparations were made with Potter homogenizer.

	Per cent of total acid phosphatase activity \pm SEM (in 0.25 M sucrose)			
	Free activity		Unsedimentable activity	
	No. of Exp.	Per cent	No. of Exp.	Per cent
Homogenates	31	16.8 \pm 0.58	3 (20)*	6.0 \pm 0.65 (7.18 \pm 0.18)*
Cytoplasmic extracts	27	18.5 \pm 0.74	18	11.1 \pm 0.40
All preparations	58	17.3 \pm 0.21	—	

* From Beaufay et al. (5)

minations. There is no evidence of the 10% increase in acid phosphatase observed by Berthet (6) 1 or 2 hr after injection of glucagon. It must be noted that this author gave 100 μ g of glucagon per 100 g body weight, i.e. twice the amount administered in our experiments. The increase in cathepsin D activity reported by Berthet (6) has been confirmed. Acid deoxyribonuclease activity was not affected by glucagon and no change in the hepatic cytochrome oxidase or glucose-6-phosphatase activities was observed.

Influence of Glucagon on Fragility of Lysosomes

MECHANICAL FRAGILITY: In confirmation of the observations reported by Berthet (6), glucagon administration was found to cause a significant increase in the free and unsedimentable acid phosphatase activities of homogenates and cytoplasmic extracts. Fig. 2 summarizes these results, which are plotted in terms of the differences observed between glucagon-treated animals and their littermate controls, killed at the same time after injection. There was no indication that the sham injection had any significant effect on the state of acid phosphatase. As shown in Table II, the free activities were essentially the same in cytoplasmic extracts and in homogenates; accordingly, the results obtained on both types of preparations have been pooled in Fig. 2. Unsedimentable activity values are plotted separately for homogenates and for cytoplasmic extracts, since, as shown in Table II, the proportion of unsedimentable acid phosphatase was lower in the former than in the latter preparations, possibly

TABLE III

Influence of Homogenization Method on Free Acid Phosphatase Activity of 0.25 M Sucrose Homogenates

Results shown are averages of 12 experiments (Two after 1½ hr and ten after 2 hr) in which the livers of the glucagon-treated animal and of its littermate control were divided into two pieces, one of which was homogenized with the Potter, the other with the Dounce homogenizer. The results were analyzed statistically by 2 \times 2 factorial variance analysis. Differences between means were evaluated by the Student-Newman-Keuls multiple range test.

	Free acid phosphatase, per cent of total activity (in 0.25 M sucrose)		
	Potter	Dounce	Difference (P-D)
Control	14.3	10.6	3.7*
Glucagon	17.0	12.1	4.9*
Difference (G-C)	2.7*	1.5*	1.2

* P < 0.01.

because the extracts were centrifuged at a higher dilution than the homogenates.

As shown in Fig. 2, the change in accessibility and distribution of acid phosphatase takes place rather suddenly after a time-lag of about 30 min and reaches its maximum 45 min after injection. On an average, an additional 7% of the total activity is unmasked at this stage, appearing entirely in the soluble fraction both in homogenates and in cytoplasmic extracts. The changes

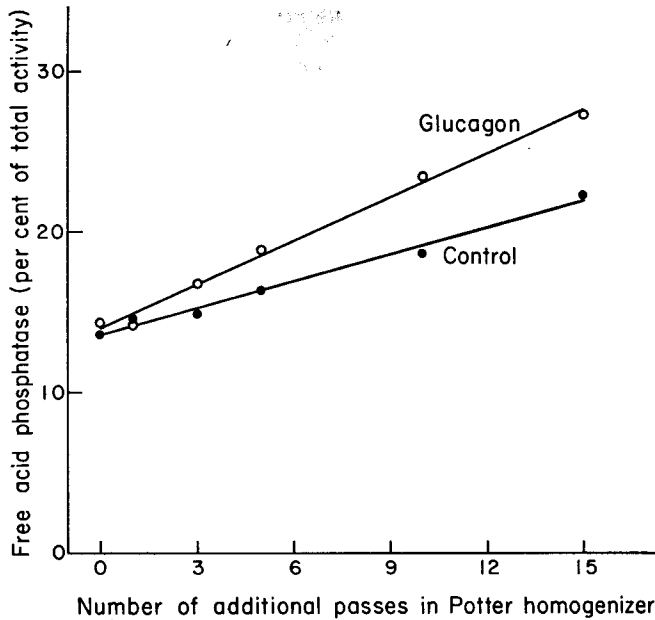


FIGURE 3 Influence of glucagon on mechanical fragility of hepatic lysosomes. The livers from glucagon-injected rats and from their littermate controls were removed 1½ hr after injection and homogenized with the Dounce homogenizer. They were then rehomogenized in the Potter homogenizer. Free and total acid phosphatase activities were measured after the number of additional homogenizations shown on the abscissa scale. Results are averages from three experiments. Regression lines were fitted to the points and found to differ significantly in slope ($P < 0.001$).

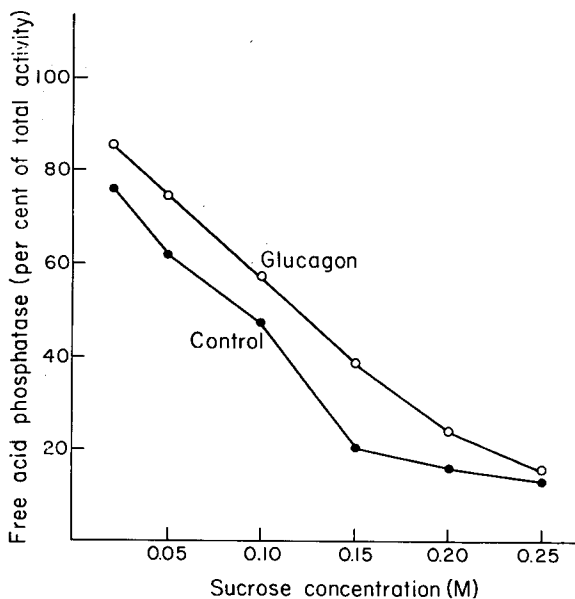


FIGURE 4 Influence of glucagon on osmotic fragility of hepatic lysosomes. The livers from glucagon-injected rats and from their littermate controls were removed 1½ hr after injection and homogenized with the Dounce homogenizer. Samples of the homogenates were diluted to the sucrose concentration shown on the abscissa scale and kept at 0° for 30 min. The sucrose concentration was then restored to 0.25 M and free and total acid phosphatase activities were measured. Results are averages from four experiments. Factorial variance analysis indicates that the response to changes in sucrose concentration is significantly different in lysosomes from glucagon-treated animals ($P < 0.005$).

observed last for at least 2 hr after injection, but the further time-course of the phenomenon is not entirely clear. According to the limited data shown in Fig. 2, the free activity appears to fall back to normal at the end of about 4 hr, whereas the unsedimentable activity seems to remain elevated for more than 6 hr (Average of seven difference values observed at 4 hr or later =

$+ 6.0 \pm 0.93$). If these results are correct, they would mean that at 4–6 hr after injection of glucagon the difference between free and unsedimentable activity observed in normal animals (Table II) would be largely obliterated. Additional determinations will have to be made to check this point.

In order to find out whether the lysosomal

TABLE IV
Free Acid Phosphatase Activity of Control Preparations
Exposed to 0.15 M Sucrose

Values, which are pooled from animals killed at times varying between 0 and 4 hr after injection of solvent, represent free acid phosphatase activity observed in preparations after a 30-min exposure to 0.15 M sucrose as explained in legend to Fig. 4. All preparations were made with Potter homogenizer. From corresponding values in 0.25 M sucrose given in Table II, it may be calculated that osmotic shock releases an additional 17.7% of enzyme activity in homogenates and an additional 19.0 in cytoplasmic extracts. This difference in the amount of enzyme released is not significant.

Preparation	No. of Exp.	Free acid phosphatase, per cent of total activity \pm SEM (in 0.15 M sucrose)
Homogenates	22	34.5 \pm 0.93
Cytoplasmic extracts	27	37.5 \pm 1.25
All preparations	49	36.6 \pm 0.80

rupture induced by glucagon administration takes place in the intact animal or during homogenization, homogenates of parts of the same livers were prepared with the Potter and Dounce homogenizers. According to the results of Bowers et al. (7) on spleen, the latter device is less injurious to fragile particles than the former. As shown by the results of Table III, such is also the case for liver. On an average, a smaller effect of glucagon was observed in the Dounce than in the Potter homogenates. However, when Dounce homogenates were subjected to additional passes in the Potter homogenizer, acid phosphatase was released more readily in preparations from glucagon-treated animals than in the control homogenates (Fig. 3). It appears from these results that glucagon renders the hepatic lysosomes, or at least some of them, more sensitive to mechanical injury. Actual intracellular release of lysosomal enzymes probably does not occur after injection of the hormone.

OSMOTIC FRAGILITY: As shown in Fig. 4, administration of glucagon 1½ hr before killing resulted in a distinct increase in the amount of acid phosphatase set free by a 30-min exposure of Dounce homogenates to decreasing sucrose concentrations. The effect of glucagon shows up particularly well in 0.15 M sucrose and this

concentration was chosen for a more detailed time study of the phenomenon. Potter homogenates or cytoplasmic extracts were used in these experiments. Both preparations exhibited the same average degree of acid phosphatase release in the control (Table IV) and experimental series at all times after injection. The sham injection had no influence on the osmotic fragility of the particles. The results obtained on the glucagon-treated animals are shown in Fig. 5. It is seen that the increase in osmotic fragility of the lysosomes takes place fairly suddenly around the 30th min after injection and is largely abolished about 1 hr later. During the peak period, an additional 10% of the total enzyme activity is released by the osmotic

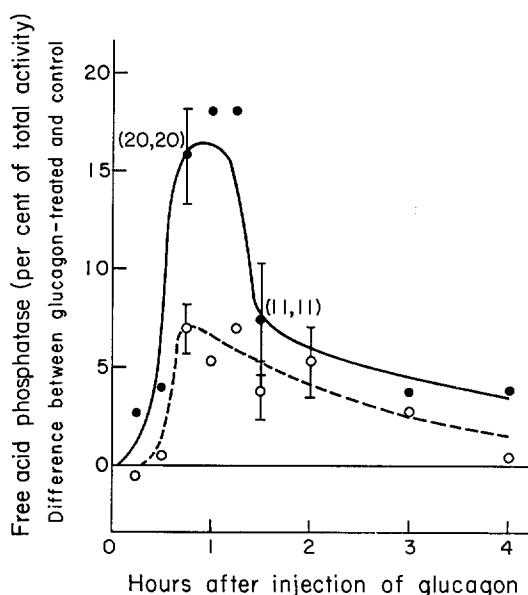


FIGURE 5 Effect of glucagon on osmotic fragility of hepatic lysosomes. Potter homogenates or extracts were prepared at various times after injection of glucagon or solvent, exposed for 30 min to 0.15 M sucrose, and analyzed for free and total activities of acid phosphatase as in experiments shown in Fig. 4. Solid circles are averages of differences observed between glucagon-treated animals and littermate controls, as in Fig. 2. Larger series are shown with their standard error of mean and, in parentheses, the number of control and glucagon-treated animals. Values obtained on homogenates and cytoplasmic extracts did not differ significantly and were pooled. Altogether 44 pairs of values were used to construct the graph. Open circles and the dotted line are taken from Fig. 2 and represent differences in free activity observed in preparations not subjected to an osmotic shock.

shock, over and above the amount set free by Potter homogenization.

An additional series of experiments was performed on Dounce homogenates from animals killed 1½ hr after injection of either glucagon or

solvent. As shown in Table V, the increased osmotic fragility of the lysosomes induced by glucagon can be seen in Dounce as well as Potter homogenates. There is even an indication that more particles are disrupted by the osmotic shock in Dounce homogenates, as though most mechanically fragile particles that had been spared by the use of a gentler homogenization procedure suffered subsequent disruption in 0.15 M sucrose. As will be pointed out in the Discussion, such a relationship between osmotic and mechanical fragility is understandable if both are due to the same cause, such as an increase in the size of the particles. However, a similar relationship does not hold true in the control preparations. As shown by comparison of the data of Tables III and V, considerably less acid phosphatase activity is unmasked by osmotic shock in Dounce homogenates from normal animals than in Potter homogenates. A more vigorous homogenization seems to render particles susceptible to osmotic shock. Whatever the mechanism of this effect, it is not seen after treatment with glucagon.

Influence of Glucagon on Sedimentation Properties of Lysosomes

In each of the seven experiments to be described in this section, a glucagon-treated rat and its littermate control injected with solvent were killed at times ranging between 0 and 4 hr after injection;

TABLE V
Influence of Homogenization Method on Free Acid Phosphatase of Homogenates Exposed to 0.15 M Sucrose

Results shown are averages of comparative experiments on Potter homogenates (six values) and on Dounce homogenates (seven values) subjected to 0.15 M sucrose for 30 min. Animals were killed 1½ hr after injection of glucagon or of solvent. Different animals were used in two series. The results were analyzed statistically by 2 × 2 factorial variance analysis. Differences between means were evaluated by the Student-Newman-Keuls multiple range test.

Treatment	Free acid phosphatase activity, per cent of total (0.15 M sucrose)		Difference (P-D)
	Potter	Dounce	
Control	36.6	23.5	13.1†
Glucagon	46.6	39.5	7.1§
Difference (G-C)	10.0*	16.0‡	-6.0

* P < 0.05.

† P < 0.01.

§ P > 0.05.

TABLE VI
Enzyme Recoveries in Sedimentation Experiments
Values are means of seven experiments ± standard error.

Enzyme	Per cent of total activity					
	Nuclear fraction*		Recovery (gradient)‡		Recovery (high-speed)‡	
	Control	Glucagon	Control	Glucagon	Control	Glucagon
Cytochrome oxidase	13.5±1.9	11.7±0.65	82.0±3.7	76.3±4.0	88.6±2.5	86.3±3.4
Glucose-6-phosphatase	5.0±0.66	4.5±0.20	97.5±3.8	94.1±2.7	86.7±6.0	84.8±6.5
Acid phosphatase	3.3±0.03	3.5±0.02	104.4±1.3	101.4±2.5	100.7±2.0	98.9±2.8
Cathepsin D	5.7±0.68	5.7±0.60	108.7±1.4	109.3±2.3	113.2±3.2	106.7±2.0
Acid deoxy-ribonuclease	4.4±0.43	6.3±0.75	102.3±1.4	107.2±2.3	106.1±2.0	114.2±1.2

* Per cent of sum of activities of nuclear fraction and of cytoplasmic extract.

‡ Per cent of activity in cytoplasmic extract. Gradient refers to the fractions separated by density gradient centrifugation; high-speed to the pellet and supernatant separated by centrifuging extract for 45 min at 100,000 g for evaluation of unsedimentable activity.

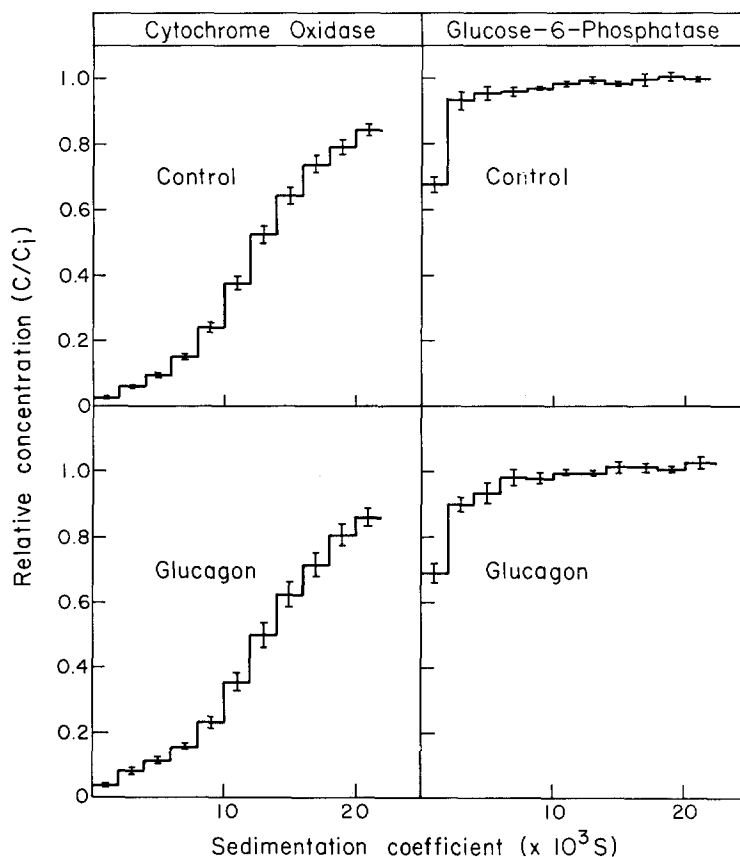


FIGURE 6 Sedimentation boundaries of cytochrome oxidase and glucose-6-phosphatase in hepatic cytoplasmic extracts from glucagon-treated animals and their littermate controls. Diagrams represent pooled values (means \pm standard error) from seven experiments performed on animals killed at 0, 30, 45, 60, 90, 150, and 240 min after injection of glucagon or solvent. C_i and C refer to enzyme concentrations before and after centrifugation, respectively.

their livers were treated according to the procedure outlined in Fig. 1. Neither the injection nor the time of killing had any effect on the partition of enzymes between the nuclear fraction and the cytoplasmic extract. The pooled values observed in each group are listed in Table VI, which also gives the over-all enzyme recoveries for the fractionations.

The seven control animals gave similar results and pooled sedimentation diagrams for this group are given in Figs. 6 and 7. Glucagon injection was without significant influence on the sedimentation properties of cytochrome oxidase and glucose-6-phosphatase and the results obtained for these enzymes in glucagon-treated animals were also pooled. They are shown in Fig. 6.

The manner in which the sedimentation diagrams of the acid hydrolases were analyzed is illustrated in Fig. 7. The unsedimentable activities measured on the high-speed supernatant were first plotted on the diagrams, to indicate the partition between particle-bound and soluble enzyme in each fraction. When smooth curves were fitted to the histograms and extrapolated to the origin, the relative concentration reached was found to be distinctly higher than the base line provided by the unsedimentable activity, especially for acid phosphatase. This difference was defined as the *slow component* and evaluated graphically as shown in Fig. 7. A *fast component*, comprised of all completely sedimented particles, was also defined; it was evaluated from the plateau value of the sedimenta-

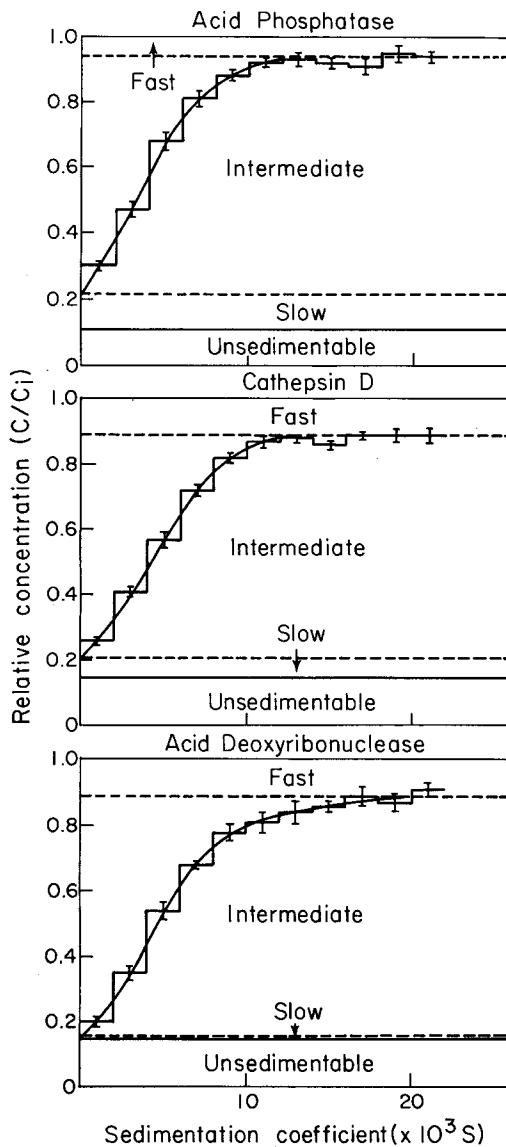


FIGURE 7 Sedimentation boundaries of acid hydrolases in hepatic cytoplasmic extracts from control animals. Diagrams represent pooled values (means \pm standard error) from seven experiments performed on animals killed at 0, 30, 45, 60, 90, 150, and 240 min after injection of solvent. Lower horizontal line gives relative concentration of enzyme in high-speed supernatant (unsedimentable activity). Broken lines indicate slow, intermediate, and fast components of the population. C_i and C refer to enzyme concentrations before and after centrifugation, respectively.

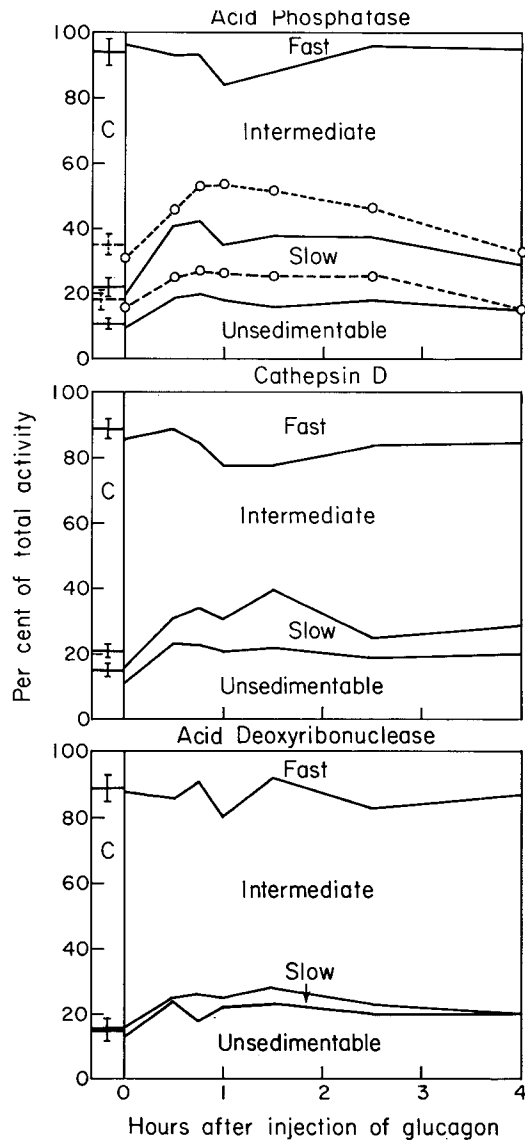


FIGURE 8 Influence of glucagon on sedimentation properties of acid hydrolases in hepatic cytoplasmic extracts. For method of evaluating the slow, intermediate, and fast components, see Fig. 7. Broken lines on acid phosphatase diagram show free activity in 0.25 M sucrose (lower curve) and after exposure to 0.15 M sucrose (upper curve). Levels shown in column on left hand side are means \pm standard deviation of pooled values obtained on controls.

tion diagram. The *intermediate component* was estimated by difference. Finally, the *median sedimentation coefficient* was obtained from the abscissa value corresponding to the point on the boundary curve situated midway between the ordinate value of 1.0 and the base line of unsedimentable activity. The same estimate was also made on the cytochrome oxidase diagrams (for which the base line is zero). The sedimentation of glucose-6-phosphatase was insufficient to permit an estimate of median sedimentation coefficient for this enzyme.

The enzyme distributions and sedimentation coefficients obtained for the three acid hydrolases in glucagon-treated animals are shown in Fig. 8 and in Table VII. The free acid phosphatase values plotted in Fig. 8 indicate a response to glucagon possibly somewhat more prolonged than the average response depicted in Fig. 5. The unsedimentable acid phosphatase activity is increased and the other two hydrolases share this increase. It will be noted that after 4 hr the free and unsedimentable acid phosphatase activities are equal. This has never been seen in normal extracts; the possibility that it may reflect a late consequence of the action of glucagon has been mentioned above.

The modifications in sedimentation properties undergone by the particulate hydrolases under the influence of glucagon are complex. All three enzymes exhibit a significant rise in median sedimentation coefficient (Table VII) which can-

TABLE VII
Median Sedimentation Coefficients

Median sedimentation coefficients were estimated from boundary curves at a relative concentration midway between 1.0 and the base-line of unsedimentable activity. Differences between means were evaluated by *t* test.

Enzyme	Average median sedimentation coefficient ($\times 10^5$) between 0.5 and 2.5 hr. after injection		
	Control	Glucagon	Difference
Cytochrome oxidase	12.8	12.9	0.1*
Acid phosphatase	3.9	5.1	1.2†
Cathepsin D	5.1	7.1	2.0‡
Acid deoxyribonuclease	5.1	7.4	2.3§

* $P > 0.10$.

† $P < 0.05$.

§ $P < 0.01$.

not be ascribed to improper control of the centrifugation conditions, since a similar increase in the median sedimentation coefficient of cytochrome oxidase was not observed. A slight, though insignificant in itself, increase in the fast component (Fig. 8) reflects this change in sedimentation behavior. In addition, there is an increase in the slow component, particularly marked for acid phosphatase, less so for cathepsin D and almost negligible for acid deoxyribonuclease. As a result of these changes, the lysosomal population shows a tendency to become bimodal and to divide into a slowly sedimenting component and a much faster moving component. The magnitude of the increase in median sedimentation coefficient depends on the relative contribution of these two components to the total particle population: it is greater for the enzymes showing a smaller increase in the slowly sedimenting component (Table VII). Changes in sedimentation properties of the lysosomes follow the same time-course as the changes in their fragility and, except for the unsedimentable activities, all measured parameters approach control values after 4 hr.

DISCUSSION

The response of hepatic lysosomes to large doses of glucagon appears to be characterized by a series of more or less synchronous changes, starting fairly suddenly about 30 min after injection and reaching a peak 15–30 min later. The changes that have been observed are: 1) an increase in mechanical fragility leading to the appearance of a higher proportion of acid phosphatase, cathepsin D, and acid deoxyribonuclease in free unsedimentable form; 2) an increase in osmotic sensitivity, as revealed by measurements of free acid-phosphatase activity after exposure to decreasing concentrations of sucrose; 3) a change in the distribution of sedimentation coefficients within the population, characterized on the one hand by a small increase, particularly evident for acid phosphatase, in the amount of enzyme associated with slowly sedimenting particles, and on the other by a distinctly larger increase in the amount associated with particles of higher sedimentation coefficient. All these changes, except possibly the increase in unsedimentable activity, have nearly disappeared 4 hr after injection of the hormone.

The simplest explanation that would account for most of these effects is to assume that many of the hepatic lysosomes increase in size as a

result of glucagon administration. In the cases of cathepsin D and acid deoxyribonuclease, which are least affected by an increase in the slow component, the increment in median sedimentation coefficient is of the order of 50%. If the particles are spherical and their density and shape remain unaltered, this would correspond, on an average, to a 23% increase in diameter, a 50% increase in surface area and an 80% increase in volume.

In a homogenizer, particles are damaged mostly by shearing forces generated by the velocity gradient and by compression between the walls of the tube and pestle. Both effects increase with the size of the particle and thus the enhanced mechanical fragility of the lysosomes could be related to an increase in their size. Similarly, their enhanced osmotic sensitivity could also be a reflection of increased particle size, since the stress on the outer membrane of a spherical particle imposed by a given excess of inner over outer osmotic pressure is proportional to the particle radius. It is, of course, possible that other physical parameters, such as the density of the particles, their internal osmotic pressure, or the cohesiveness of their surrounding membrane, are affected by glucagon. If an increase in size is the main factor responsible for the observed changes, a large proportion of the hepatic lysosomes must be so affected, since those contributing to the unsedimentable activity, to the fast component, and to the intermediate component (Fig. 8 and Table VII) all appear to be involved.

A question which must await further studies concerns the possible relationship between the physical changes observed in hepatic lysosomes and the simultaneous appearance of numerous autophagic vacuoles in the livers of glucagon-treated animals. It is tempting to postulate a direct correlation between the two phenomena. An increase in the size of at least part of the lysosomes would be consistent with the morphological observations which suggest that pericanalicular dense bodies decrease in number as autophagic vacuoles, many of them of larger size, become more numerous (P. Baudhuin, personal communication).

At present, it is not possible to offer a satisfactory explanation for the increase in the slow component, especially since the magnitude of this change varies from one enzyme to the other. Preliminary experiments have shown that the acid phosphatase associated with this component forms

a boundary in higher centrifugal fields and is at least 50% latent, suggesting that it is associated with true lysosomal particles. It is possible that small lysosomes appear as an outcome of the fusion processes believed to be involved in autophagic-vacuole formation, or that primary lysosomes are formed as a result of glucagon injection, perhaps in the form of Golgi vesicles. The latter interpretation would fit with the observations of Berthet (6) which indicate that glucagon treatment increases the total acid phosphatase and cathepsin D activities of the liver by about 10%, the average magnitude of the increase manifested by the slow component. An increase in cathepsin D activity has been confirmed in our experiments, but we have been unable to find any sign of a similar increase in acid phosphatase, the enzyme showing the largest increment in slow component, or in acid deoxyribonuclease.

It is interesting that most of the observed effects do not last more than 3-4 hr. Morphological observations should reveal whether the signs of enhanced autophagy also have disappeared after this time. If so, the correlation between the two types of findings would become more secure and the quantitative results obtained in the present experiments would provide valuable information concerning the time-course of autophagy and intracellular digestion.

Whether the enhancement of cellular autophagy described by other workers and the presumably related alterations in the physical properties of the lysosomes reported in the present paper have any bearing on the physiological role of glucagon is a question which we would forbear to discuss until the influence of glucagon dosage on these effects has been investigated. The doses used here are clearly unphysiological, but were adopted because they were known to be effective in stimulating autophagy. Our main aim has been to find out whether this cellular phenomenon, which is undoubtedly of physiological significance, is accessible to biochemical investigation.

This work has also provided more accurate data on the sedimentation-coefficient distribution of cytoplasmic particles. The interpretation of the observed distribution diagrams and their relation to similar quantitative data obtained with other methods will be examined in another paper.

We are indebted to Dr. O. K. Behrens from the Lilly Research Laboratories for his generous gift of glucagon, to Drs. J. Berthet and P. Baudhuin for their

valuable advice and suggestions, and to Miss Nancy Chew and Miss Rae Liebelson for excellent technical help. One of us (R.L.D.) holds a Public Health Service Fellowship (1-F2-CA-29, 337-01) from the Na-

tional Cancer Institute. These investigations were supported by a grant from the National Science Foundation (No. GB-2871).

Received for publication 6 September 1966.

REFERENCES

1. APPELMANS, F., and C. DE DUVE. 1955. Tissue fractionation studies. 3. Further observations on the binding of acid phosphatase by rat-liver particles. *Biochem. J.* **59**:426.
2. ASHFORD, T. P., and K. R. PORTER. 1962. Cytoplasmic components in hepatic cell lysosomes. *J. Cell Biol.* **12**:198.
3. BEAUFAY, H., D. S. BENDALL, P. BAUDHUIN, R. WATTIAUX, and C. DE DUVE. 1959. Tissue fractionation studies. 13. Analysis of mitochondrial fractions from rat liver by density-gradient centrifuging. *Biochem. J.* **73**:628.
4. BEAUFAY, H., P. JACQUES, O. Z. SELLINGER, J. BERTHET, and C. DE DUVE. 1964. Tissue fractionation studies. 18. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. *Biochem. J.* **92**:184.
5. BEAUFAY, H., E. VAN CAMPENHOUT, and C. DE DUVE. 1959. Tissue fractionation studies. 11. Influence of various hepatotoxic treatments on the state of some bound enzymes in rat liver. *Biochem. J.* **73**:617.
6. BERTHET, J. 1964. Influence of glucagon on some lysosomal enzymes, Abstracts, 6th International Congress of Biochemistry, New York. 644.
7. BOWERS, W. E., J. T. FINKENSTAEDT, and C. DE DUVE. 1967. Lysosomes in lymphoid tissue. I. The measurement of hydrolytic activities in whole homogenates. *J. Cell Biol.* **32**:325.
8. CLARK, S. L. 1957. Cellular differentiation in the kidneys of newborn mice studied with the electron microscope. *J. Biophys. Biochem. Cytol.* **3**:349.
9. DE DUVE, C., J. BERTHET, and H. BEAUFAY. 1959. Gradient centrifugation of cell particles. Theory and application, *Progr. Biophys. Biophys. Chem.* **9**:325.
10. DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60**:604.
11. DE DUVE, C., and R. WATTIAUX. 1966. Functions of lysosomes. *Physiol. Rev.* **28**:435.
12. DOUNCE, A. L., R. F. WITTER, K. J. MONTY, S. PATE, and M. A. COTTONE. 1955. A method for isolating intact mitochondria and nuclei from the same homogenate, and the influence of mitochondrial destruction on the properties of cell nuclei. *J. Biophys. Biochem. Cytol.* **1**:139.
13. GIANETTO, R., and C. DE DUVE. 1955. Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β -glucuronidase and cathepsin by rat-liver particles. *Biochem. J.* **59**:433.
14. POTTER, V. R., and C. A. ELVEHJEM. 1936. A modified method for the study of tissue oxidations. *J. Biol. Chem.* **114**:495.
15. STEEL, R. G. D., and J. H. TORRIE. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York, Toronto, and London.