CHANGES IN "DROPLET" FRACTIONS FROM RAT KIDNEY CELLS AFTER INTRAPERITONEAL INJECTION OF EGG WHITE*

By WERNER STRAUS, PH.D.

(From the Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York)

(Received for publication, June 10, 1957)

In a previous report (1), the isolation of 3 "droplet" fractions from kidney cells of normal rats was recorded. Fraction I contained droplets with diameters from 1 to 5 μ, fraction II from 0.5 to 1.5 μ, and fraction III from approximately 0.1 to 1.0 μ. All three fractions showed 10 to 15 times higher activity for acid phosphatase, acid ribonuclease and desoxyribonuclease, cathepsin and β-glucuronidase than the total homogenate and the mitochondrial fraction. It was pointed out (1) that the enzymatic properties of the kidney droplets resembled those of "lysosomes," isolated from liver cells by de Duve *et al.* (2), and it was suggested (1, 3) that the kidney droplets are related to secretory granules[1] and Golgi bodies and distinct from the mitochondria.

Many investigators have attributed to the droplets from kidney cells a function in either the reabsorption, secretion, storage, or metabolism of foreign or native proteins (4–13) (see the review by Rather (14)). According to Oliver *et al.* (10, 11), proteins which have passed the glomerulus are reabsorbed by the cells of the middle portion of the convoluted tubules where they are concentrated within the droplets. Straus and Oliver (15) showed that droplets isolated after injection of egg white contained several times as much serologically reacting egg white as the other fractions of the kidney homogenate. However, Mayersbach and Pearse (16), using histochemical methods with fluorescent antibodies, could not confirm the concentration of egg white within the droplets. As will be reported later (17), the ability of the droplets to segregate proteins has been reexamined by a new, quantitative method and could be confirmed definitely.

In the present investigation, another effect of the injection of a foreign protein on the kidney droplets has been studied. It will be shown that after injection of egg white characteristic changes occur in the size, number, and enzyme

* This investigation was supported by research grants from the National Cancer Institute, United States Public Health Service (No. C-2485), and from the New York Heart Association.

[1] There has existed a long standing controversy as to whether the kidney excretes material by "secretion" from the blood through the tubule cells without passing the glomerulus, or by "filtration" through the glomerulus. When the droplets are compared in this paper with secretory granules from other cells, no connection with this controversy is implied.

content of the droplets, and that there is a concomitant release of a portion of their enzymes. This points to a possible function in the intracellular "secretion" of enzymes and supports the hypothesis of the relationship of the droplets to secretory granules.[1]

Materials and Methods

Male rats of the Sherman strain, 350 to 450 gm. in weight, were used. The animals, fed with Purina chow and water ad libitum, were injected intraperitoneally with a mixture of 7 ml. of egg white and 7 ml. of saline. The kidneys were removed 18 hours later under ether anesthesia and chilled immediately. All subsequent manipulations were done at temperatures not higher than 5°. The medullae and capsules were discarded and only the cortices used. Kidneys from untreated animals of the same age were removed at the same time and served as controls in parallel experiments. The tissue was homogenized in 30 per cent sucrose solution (18) (adjusted to pH 8.5 with NaOH) in a glass homogenizer with a motor driven pestle. In order to avoid possible alteration of the droplets by too drastic treatment, the homogenization of the tissue was interrupted after 5 up and down strokes with a loosely fitted teflon pestle (2100 R.P.M.) in a smoothwalled glass vessel, and the non-homogenized residue, separated by low speed centrifugation, was rehomogenized once more in the same way. Approximately 20 per cent of the tissue remained non-homogenized.

Fractionation of the Homogenates.—The homogenates from rats injected with egg white and from control rats were fractionated simultaneously. The procedure was similar to that used in previous experiments (1). The sediments were not washed since speed of separation and reduced manipulations were considered more important than continued purification.

Fraction NDrI, obtained by centrifugation of the homogenate for 20 minutes at 650 *g*, contained nuclei and large droplets (1 to 5 μ diameter) and was contaminated with whole cells and large mitochondria. Droplet fraction DrII, prepared by centrifuging the supernatant fluid of fraction NDrI for 1 minute at 15,000 *g*, contained droplets of intermediate size (0.5 to 1.5 μ diameter) and was contaminated by nuclei and mitochondria. Droplet fraction DrIII and the mitochondrial fraction (M) were sedimented together by centrifuging the supernatant fluid of fraction DrII for 20 minutes at 15,000 *g*. The upper mitochondrial layer was separated, with the aid of a glass rod and repeated decantation, from the dark brown lower portion of the sediment which contained the small droplet fraction DrIII (0.1 to 1.0 μ estimated diameter). Since no complete separation of the two fractions was possible in this way, droplet fraction DrIII was contaminated with many mitochondria, and the mitochondrial fraction was contaminated with small droplets. The microsomal sediment (P) was prepared by centrifuging the supernatant fluid of the mitochondrial fraction, after dilution with water 1:1, for 2 hours at 18,000 *g*. The dilution of the sucrose in the suspension from 30 to 15 per cent made it possible to attain a more complete separation of the microsomes from the final supernatant fluid (S) than in the previous experiments (1). Aliquots of the original homogenates and the non-homogenized residues were retained. All sediments were resuspended in distilled water.

In some of the experiments shown in Table II, minor changes from this scheme of fractionation were tried out. However, in all experiments, the separation of fractions from treated and from control animals was carried out during the same runs in the centrifuge, thus applying the same centrifugal forces to both. Equal amounts of kidney tissue, pooled from two to three rats, from both the treated and control animals were used and fractionated with equal volumes of sucrose solution. An attempt was made to obtain approximately the same percentage of nitrogen in corresponding fractions from treated and control animals. This was achieved in most cases. Occasionally, however, differences of between 10 to 15 per cent of N in correspond-

ing fractions could not be avoided for reasons mentioned subsequently. Because of this and in order to facilitate the comparison of the enzymatic activities of corresponding fractions, a calculated average N content was taken as reference for the calculation of the enzymatic activities. If, for example, droplet fraction III contained 7.9 per cent of N of the homogenate from treated animals, and 7.5 per cent from the controls, a mean of 7.7 per cent was taken as reference for both. For the same reasons, the sum of the enzyme content of all six fractions was counted as 100 per cent. The recoveries actually found are indicated in the last column of Table II. These adjustments correct for slight differences in experimental conditions between the corresponding fractions from treated and control animals. For example, those differences due to decantation from the sediments or in the separation of fraction DrIII from M (see above and under Section *B*) are minimized in the calculation of the results and mainly differences of enzyme content are expressed (Tables I and II).

Counting of Droplets in Fraction NDrI.—Droplets were counted in the Petroff-Hausser bacterial counting chamber after suitable dilution of the suspensions, either under a dry objective (magnification 60 × 10) or under oil immersion (magnification 90 × 7). If the diaphragm of the microscope was kept open and the microscope tube focussed up and down, the droplets could be easily distinguished from contaminating mitochondria by their higher refractility. In addition, the mitochondria tended to swell after some minutes at room temperature in the counting chamber and then appeared very pale or became invisible (with open diaphragm), whereas the droplets showed much greater stability and did not change their appearance and strong light refraction.

When partially purified large droplets (1 to 5 μ diameter) of fraction NDrI were counted and the enzymatic activities correlated with the number of droplets, relatively reproducible results were obtained in different experiments. When very impure suspensions of droplets or those containing many small droplets were counted, the results fluctuated to some extent. In most experiments, the large droplets of fraction NDrI were therefore partially purified before being counted.

The partial purification of large droplets of fractions NDrI for the experiments shown in Table III was carried out in the following way. The kidney homogenates, after separation of whole cells, were centrifuged for 20 minutes at 650 *g*. The main nuclear layer of the sediment was removed by swirling repeatedly with sucrose solution and decanting. The thin brown bottom portion remaining in the centrifuge tube and containing large droplets in concentrated form, was resuspended in 30 per cent sucrose solution. In some experiments, these droplets were purified further by repeating the differential centrifugation. In one experiment, only the very large droplets formed after treatment with egg white were isolated by centrifugation for 5 minutes at 500 *g*.

Enzyme Assays.—The enzymes were assayed as indicated previously (1). The following minor changes were made. In the test for RNAase, 1.25 mg. of sodium nucleate (Schwarz Laboratories, Inc., New York) were used as substrate, and in the test for β-glucuronidase, 0.15 ml. of a 5 per cent solution of Triton X (Rohm & Haas Company, Philadelphia) was added to the reagent mixture as recommended by Wattiaux and de Duve (19). All enzyme determinations were performed in duplicate.

The enzymatic activities (Tables III and IV) were expressed in the following way: *Acid phosphatase*, milligram of P liberated per 15 minutes (from β-glycero-phosphate) per milligram N. *Ribonuclease and desoxyribonuclease*, increase in optical density at 260 mμ per 0.1 milligram N during incubation for 30 minutes at pH 5.0. *Beta-glucuronidase*, phenolphthalein (5 μg. = 100 units) liberated from phenolphthalein-glucuronic acid per 30 minutes per milligram N. *Cathepsin*, milliequivalent of tyrosine per milligram N per minute, with hemoglobin as substrate. *Cytochrome oxidase*, units of red pigment formed from dimethyl-*p*-phenylenediamine per minute per milligram N. All incubations took place at 37°.

Changes in Kidney Fractions after Injection of Egg White:

A. Changes in Aspect of Sediments.—It was mentioned that during differential centrifugation of kidney homogenates droplets of different sizes are concentrated at the bottom of the sediments in dark brown layers. This sedimentation property which indicates the high density of the kidney droplets, has facilitated the purification of droplets of fraction II (0.5 to 1.5 μ diameter) and of fraction III (0.1 to 1.0 μ estimated diameter) (1, 3). It can also be used to concentrate droplets of fraction I (1 to 5 μ diameter). As a routine procedure for the separation of fraction NDrI, the kidney homogenate, after separation of whole kidney cells and blood cells, was centrifuged for 20 minutes at 650 *g* (see under *Methods*). A dark brown layer containing many large droplets (1 to 5 μ diameter) was found at the bottom of the sediment, while the upper main layer contained nuclei contaminated with droplets and mitochondria. If the supernatant fluid was centrifuged for 1 minute at 15000 *g*, and the supernatant fluid of this for 20 minutes at 15000 *g*, the dark brown lowest layers of both sediments (fractions DrII and DrIII, respectively) again contained droplets in concentrated form. Thus the amount of droplets present in fractions NDrI, DrII, and DrIII could be roughly estimated from the thickness of the dark brown layer at the bottom of each sediment.

During the isolation of droplets from normal animals and from those injected with egg white (1, 3), it was consistently observed that the thickness of the brown layer occurring at the bottom of the sediment of fraction NDrI (large droplets) appeared increased after injection of egg white, whereas that of fraction DrIII (small droplets) appeared considerably decreased. The brown layer of fraction DrII showed either a small decrease or no visible change. These differences in the sediments were found again in the present investigation in which the fractionation of kidney homogenates from rats treated with egg white and from control animals was carried out under practically the same experimental conditions (see under *Methods*). On the basis of these observations, it was decided to determine whether the changes in the aspect of the sediments could be correlated with differences in the distribution of the enzymes.

B. Changes in Enzyme Content of Isolated Fractions. —Kidney homogenates from rats treated with egg white and from control rats were simultaneously fractionated into fractions NDrI, DrII, DrIII, M, P, and S, as indicated under *Methods*, and the enzymes in each fraction were assayed. As can be seen from Table I, there was a marked decrease of the hydrolytic enzymes tested in fraction DrIII (small droplets) prepared from treated animals. This coincides with the visual observation described in the previous paragraph. The hydrolytic enzymes tested in fractions DrII and M were also decreased. The decrease of these enzymes in the fractions containing the small droplets (DrII, DrIII, and M) was accompanied by an increase of the same enzymes in the supernatant fluid (S), except in the case of β-glucuronidase (Table I).

Since the mitochondria have been implicated as the precursors of the droplets (11, 12, 20), cytochrome oxidase as a typical mitochondrial enzyme was assayed. However, no marked changes in the distribution of cytochrome oxidase were observed after treatment with egg white (Table I).

In Table II, the results of five experiments are summarized. For simplification, differences between the enzyme content of the cell fractions derived from

TABLE I

Change in Distribution of Enzymes in Fractions of Rat Kidney Homogenate after Injection of Egg White

Percentage values; homogenate = 100 per cent; NDrI, nuclear fraction + droplet fraction I; DrII, droplet fraction II; DrIII, droplet fraction III; M, mitochondrial fraction; P, microsomal fraction; S, final supernatant fluid. Crude fractions, separated from kidney homogenates of injected and control animals by single sedimentations during the same runs in the centrifuge.

	NDrI	DrII	DrIII	M	P	S
Nitrogen						
Treated	11.4	7.5	7.8	15.9	14.4	43.0
Control	10.2	6.9	7.7	17.0	16.0	42.2
Acid phosphatase						
Treated	18.2	9.6	10.3	12.9	18.8	30.2
Control	20.2	12.2	15.9	13.8	15.2	22.7
RNAase						
Treated	23.7	8.8	7.2	9.3	5.7	45.3
Control	25.8	12.3	11.4	10.4	4.5	35.6
DNAase						
Treated	17.7	11.9	7.0	7.0	6.2	50.2
Control	26.3	16.8	13.1	10.8	6.0	27.0
Cathepsin						
Treated	11.5	8.6	10.4	17.6	16.6	35.3
Control	14.4	10.3	16.0	21.6	14.7	23.0
β-Glucuronidase						
Treated	16.4	9.0	14.4	16.2	12.5	31.5
Control	15.0	9.4	17.3	16.0	10.9	31.4
Cytochrome oxidase						
Treated	15.5	13.8	19.8	40.5	8.5	1.9
Control	14.9	14.2	20.3	40.0	8.6	2.0

treated and from control animals are expressed as percental changes, the content of the controls being considered 100. In order to make it clear how the figures of Table II were calculated, the experiment shown in Table I is again shown in Table II (experiment number 5). In the first experiment one enzyme, in the second two, in the third three, in the fourth five, and in the fifth experiment six enzymes were assayed. As can be seen from Table II, acid phosphatase, ribonuclease, desoxyribonuclease, and cathepsin were considerably decreased after treatment with egg white in those fractions (DrII, DrIII, and M)

containing small droplets, and were considerably increased in the final supernatant fluid (S). No consistent changes were observed in fractions NDrI and P. These observations, as well as the different behaviour of β-glucuronidase, will be discussed below.

TABLE II

Shift of Enzymes in Fractions of Rat Kidney Homogenates after Injection of Egg White

Per cent decrease (−) or increase (+) of enzymes in fractions from injected animals as compared with corresponding fractions from control animals. Designation of fractions as in Table I. In last line, the average nitrogen content of fractions.

Experiment No.	Enzymes	NDrI	DrII	DrIII	M	P	S	Original recovery Treated	Control
I	Acid phosphatase	−2	−35	−40	−3	0	+29	95	97
II	Acid phosphatase	−1	−18	−35	−17	−7	+31	101	96
	β-Glucuronidase	+22	−7	−31	−3	+19	+1	93	94
III	Acid phosphatase	+8	−13	−31	−19	−3	+26	99	97
	Acid RNAase	+12	−19	−32	−16	+3	+16	96	97
	β-Glucuronidase	+7	−19	−20	−5	+3	+8	97	98
IV	Acid phosphatase	−4	−20	−23	−6	+3	+38	101	96
	Acid RNAase	+18	−19	−37	−19	−1	+25	93	94
	Acid DNAase	−1	−30	−38	−38	−21	+60	92	96
	Cathepsin	−1	−18	−39	−12	+3	+34	90	96
	β-Glucuronidase	−2	+54	−11	−4	−14	+1	98	97
V	Acid phosphatase	−10	−22	−35	−7	+16	+32	96	97
	Acid RNAase	−8	−28	−37	−10	+26	+27	96	99
	Acid DNAase	−33	−29	−47	−35	+2	+86	100	94
	Cathepsin	−20	−17	−35	−19	+12	+54	98	97
	β-Glucuronidase	+9	−5	−17	+1	+14	0	94	91
	Cytochrome oxidase	+4	−3	−3	+1	+1	—	93	94
I to V	Average nitrogen	10.4	7.0	7.5	15.1	13.5	46.5	98.5	97.5

As mentioned under *Methods*, some of the experimental conditions varied slightly, and this may have caused some of the fluctuations seen in Table II. One such factor was the size of fraction NDrI which varied with the size of the non-homogenized residue[2]; the other was the difficulty in obtaining a sharp separation of the lower, dark brown and the upper, yellow-brown layers of the "mitochondrial" sediment (fractions DrIII and M, respectively; see under *Methods*).

[2] No significant differences in the enzyme contents of the non-homogenized residues from treated and from control animals were found. These residues were always assayed along with the other fractions.

C. Changes in Enzyme Content of Average Droplet in Fraction NDrI.—Although the brown bottom layer in fraction NDrI appeared thicker after treatment with egg white, suggesting an increase in large droplets (see section *A*), no corresponding increase, but rather a decrease of the enzyme content of this fraction was observed (Table II). This seemed to indicate that the droplets of fraction NDrI contained less hydrolytic enzymes in treated than in control animals. In order to test this point, the droplets of fraction NDrI from treated and from control animals were partially purified, as described under *Methods*, the droplets counted in a bacterial counting chamber, and the enzymatic activities per average droplet determined.

Table III shows the results of four to six experiments. As may be seen from the table, the average droplet separated from fraction NDrI from animals treated with egg white showed lower enzymatic activities than the average

TABLE III

Enzyme Activities per Average Large Droplet in Normal and Egg White–Treated Rats

Activities per 10^6 droplets, counted in a bacterial counting chamber, of partially purified large droplets of fraction NDrI from injected and control rats. Means of four to six experiments. In last column, number of droplets in whole fraction NDrI, per mg. N of homogenate. Enzymatic activities expressed as indicated under *Methods*.

	Acid phosphatase	DNAase	RNAase	β-Glucuronidase	Cathepsin	Number in fraction NDrI
Treated	0.23	0.016	0.020	3.3	0.47×10^{-7}	76×10^6
Control	0.35	0.030	0.036	5.2	0.77×10^{-7}	37×10^6

droplet from the corresponding fraction from control animals. It should be noted that the partial purification required for accurate counting caused the loss of many droplets and that it was not certain to what extent the remaining ones could be considered representative of the whole fraction. As will be mentioned in a later paragraph, the average droplet in fraction NDrI from treated animals was larger than the one in the corresponding fraction from control animals, although the range of size was the same in both (1 to 5 μ diameter).

In order to test in a more quantitative manner the increase in *number* of large droplets after treatment with egg white, the droplets of the whole fraction NDrI were counted. The fractions were prepared from five injected and five control animals by single sedimentations for 20 minutes at 650 *g*. As can be seen from the last column of Table III, approximately double the number of droplets (mean values of five experiments) were counted in fraction NDrI from treated animals as in the corresponding fraction from control animals.

The increase in *size* of droplets after injection of egg white could be seen under the microscope, but was also inferred from the following observation. If a partially purified droplet suspension, prepared, as indicated under *Methods*,

from fraction NDrI from rats injected with egg white, was centrifuged for 5 minutes at 500 *g*, a sediment containing many very large droplets was found. The suspension from control animals, treated in the same way, yielded only an insignificant sediment. Low speed centrifugation had been used in previous work (3) for the isolation of the very large droplets appearing after intraperitoneal injection of egg white.

D. Changes in Enzyme Content of Whole Kidney.—The changes reported above concern the distribution of enzymes in the fractions of the kidney homogenate after treatment with egg white. The question arises whether these changes in the intracellular distribution are accompanied by changes in the enzyme content of the whole kidney. Such changes should find expression in the specific enzymatic activities (activity per mg. N) of the kidney homogenate. It is important to note in this connection that there was no significant change in the N content of the kidney after injection of egg white. Thus the N content of dry kidney substance was found to be 11.25 ± 0.13 per cent from normal rats and 11.33 ± 0.12 per cent from rats which had been injected 18 hours previously with egg white (each figure the average of four animals).

TABLE IV

Comparison of Specific Enzymatic Activities in Kidney Homogenates of Egg White-Treated (18 Hours after Injection) and Control Rats

Mean values with standard deviations. Activities expressed as indicated under *Methods*. Number of experiments in parenthesis.

	Treated			Controls		
Acid DNAase	0.46	± 0.04	(9)	0.34	± 0.04	(9)
Acid RNAase	1.12	± 0.11	(9)	0.74	± 0.05	(9)
Cathepsin	0.180	± 0.020	(7)	0.137	± 0.012	(7)
Acid phosphatase	74.7	± 2.0	(11)	74.8	± 3.1	(11)
β-Glucuronidase	1220	± 126	(7)	1252	± 136	(7)
Cytochrome oxidase	17.1	± 0.8	(10)	18.3	± 0.8	(10)

Table IV shows the specific enzymatic activities of total homogenates 18 hours after injection of egg white and of homogenates from untreated animals. As can be seen from the table, there was approximately a 30 per cent increase of ribonuclease, desoxyribonuclease, and cathepsin, no change in acid phosphatase and β-glucuronidase, and approximately a 7 per cent decrease of cytochrome oxidase, after treatment with egg white. These results will be discussed below.

E. Activation of Enzymes by Osmotic Effects.—It was shown by Appelmans and de Duve (21) and Gianetto and de Duve (22) that the enzymes of the hepatic lysosomes become active only when the sac-like structure of the granules is destroyed by me-

chanical or osmotic damage, for example by treatment in the Waring blendor, by repeated freezing and thawing, by resuspension in water instead of sucrose solution, or by incubation of the suspension in sucrose at 37°. According to the investigators mentioned (21, 22), these procedures cause the rupture of the membranes which, if present, prevent the access of the substrates to the enzymes.

It was apparent early in our work that the droplets from kidney cells must have similar properties, since the enzymes were not active as long as the droplets remained intact. Before testing their enzymatic properties, the isolated kidney droplets were therefore disintegrated by resuspension in distilled water (3). In later work (1), preincubation in buffer solution, pH 5.0, at 37° was also used.

TABLE V

Activation of Enzymes by Osmotic Effects

Crude droplet fractions from kidneys of rats treated with egg white and from control rats were incubated for 15 minutes at 37° in different concentrations of sucrose. Enzyme activities in sucrose are expressed as percent of maximum activities determined after complete disintegration of the droplets in distilled water and after preincubation.

Concentration of sucrose	Per Cent of Maximal Activity											
	Injected			Controls			Injected			Controls		
	DrI	DrI	DrI	DrI	DrI	DrI	DrII + III	DrII + III	Dr III	DrII + III	DrII + III	Dr III
	Acid Phosphatase	β-Glucuronidase	DNAase	Acid Phosphatase	β-glucuronidase	DNAase	Acid Phosphatase	β-Glucuronidase	DNAase	Acid Phosphatase	β-Glucuronidase	DNAase
0.53 M	44	42	42	37	37	36	51	37*	42	61	34*	65
0.28 M	51	53	41	41	47	38	50	38*	42	58	42*	65
0.16 M	62	56	69	57	50	57	56	45*	63	68	43*	67
0.097 M	75	74	75	72	74	73	67	57*	76	79	44*	89

* Enzyme probably inhibited.

Since de Duve and coworkers (21, 22) had already made a thorough study of the activation process in the case of the liver granules, it was not considered necessary to do the same in the present investigation. It was, however, considered pertinent to establish whether or not the injection of egg white causes changes in the osmotic properties of the droplets. If, for example, the droplets would become more fragile after the treatment with egg white, this might cause artifacts giving rise to similar changes as were observed. The question was also of interest as to whether droplets of different sizes (fractions I and III) might show differences in their osmotic properties.

Two orienting experiments were made to study these points. Droplet fractions from treated and from control animals were resuspended in 0.88 M sucrose solution. Within a few hours after preparation, 0.1 ml. of each suspension was added to incubation mixtures containing 0.5, 0.25, 0.125, and 0.062 M sucrose, respectively, and incubated for 15 minutes at 37°. The observed activities of the enzymes in these different concentrations of sucrose were compared with the maximal activities, measured after

disintegration of the droplets by dilution with water and preincubation in buffer solution, pH 5.0, at 37°. In the first experiment, the activities of acid phosphatase and β-glucuronidase in fraction NDrI and in the combined fractions DrII + DrIII (sedimented together[3] in the centrifuge) from treated and from control animals were compared; in the second experiment, the activities of acid phosphatase, DNAase, and β-glucuronidase in fractions NDrI and DrIII only, were compared.

Table V shows the essential results. It can be seen from the table, that similar osmotic effects were observed for the droplets from treated and from control animals. The degree of activation in different concentrations of sucrose was similar for acid phosphatase, DNAase, and also for β-glucuronidase in those fractions in which the activity of this enzyme was not inhibited (see next paragraph). This supports the conclusion already reached from other data that these enzymes belong to the same type of particles. The very small droplets (fraction DrIII) seem to be more fragile than the larger ones although more experiments would be required to decide this question.

Lower activities were observed for β-glucuronidase in fractions DrII + DrIII and DrIII; they were probably caused by the inhibition of the enzyme by mitochondrial fragments (2). These figures are therefore not comparable with the others. The maximal activity of β-glucuronidase was determined in the presence of Triton X (19).

DISCUSSION

The possibility of artifacts should be critically considered before any attempt is made to reconstruct events occurring *in vivo* from results obtained in experiments with isolated fractions. During the homogenization of tissues and during procedures of isolation of subcellular structures, material may be lost by diffusion or may be adsorbed to these structures. In addition, osmotic or autolytic effects may cause further disintegration. With such considerations in mind, it was discussed previously (1) that part of the enzymes may be lost from the droplets and may increase the concentration of these enzymes in the supernatant fluid. It is probable, however, that the stability of the kidney droplets is greater than might appear from the previous discussion. One indication for this was mentioned above; the droplets after having remained for 15 to 30 minutes in the bacterial counting chamber at room temperature, appeared unchanged, whereas the mitochondria were considerably swollen. Although the fractions obtained from treated and control animals had undergone the same manipulations *in vitro*, their enzymatic properties were found to be significantly different. This leads to the conclusion that the changes observed

[3] If it was not necessary to separate droplets of small and intermediate size, droplets of fraction II and fraction III were often sedimented together. This was carried out by centrifuging the supernatant fluid of fraction NDrI for 20 minutes at 15000 *g*. The dark brown layer at the bottom of the sediment contained droplets of fraction II and III "packed" together.

after treatment with egg white occurred *in vivo*, and were not artifacts produced *in vitro*. The mild procedures which were adopted (use of a loosely fitted teflon pestle in a smooth walled homogenizer vessel and separation of the fractions by single sedimentations) also tended to reduce the risks of artifacts. The possibility that the droplets from animals treated with egg white might be much more sensitive towards osmotic changes than those from normal rats could probably be ruled out by the experiments shown in Table V.

After treatment with egg white, the content of acid phosphatase, ribonuclease, desoxyribonuclease, and cathepsin was significantly *decreased* in the fractions containing the small droplets and *increased* in the supernatant fluid (Tables I and II). There was also a constant and marked decrease in the thickness of the brown layer of fraction DrIII, indicating a considerable reduction in the number of small droplets. This finding was satisfactorily correlated with the concomitant decrease in the enzyme content of the same fraction. Data reported in sections *A* and *C* suggest, in addition, that after the treatment mentioned the large droplets increased both in size and number. It may be concluded from this that the injection of egg white caused the transformation of small droplets into large droplets. Oliver (10), Oliver, MacDowell, and Lee (11), Zollinger (12), and Rhodin (20) have already observed an increase in large droplets in kidney cells, *in situ*, after injection of egg white. With the transformation of small droplets into large droplets, a portion of the hydrolytic enzymes was concomitantly released. This seemed to follow from the observation that fraction NDrI, although containing markedly increased numbers of large droplets (Table III), did nevertheless not show an increase in enzyme content (Table II), and that the average large droplet showed decreased activity after treatment with egg white (Table III). It may be suggested that at least a portion of the enzymes which were increased in the supernatant fluid were those which had been released from the droplets into the surrounding cytoplasm. However, other possibilities should also be considered. For example, some of the enzymes may have been released from the droplets into the extracellular spaces. Another factor adding to the complexity was the 25 to 35 per cent increase of ribonuclease, desoxyribonuclease, and cathepsin in the total homogenate after treatment with egg white (Table IV). This probably indicates a formation of new enzymes following the release of enzymes from the droplets. The tentative conclusion may be made that the increase of the hydrolytic enzymes in the supernatant fluid was partly caused by the release of enzymes from the droplets, partly by the formation of new enzymes. In this connection, it is interesting to note the different behaviour of acid phosphatase and β-glucuronidase from the three other hydrolytic enzymes. Beta-glucuronidase, although decreasing in content to some extent in the fractions containing the small droplets, did not increase in the supernatant fluid. The occasional marked increase of this enzyme in fractions NDrI and DrII (Table II) may indicate its shift from the small

droplets to intermediate or large sized droplets without being released. Acid phosphatase, on the other hand, although unchanged in the total homogenate, did nevertheless increase in the supernatant fluid. If our interpretation is correct, the increase of acid phosphatase in the supernatant fluid was mainly caused by the release of the enzyme from the droplets, whereas in the case of ribonuclease, desoxyribonuclease, and cathepsin, the increase in the supernatant fluid was due both to the release of the enzymes from the droplets and to the formation of new enzymes. Before any definite conclusion on these questions can be made, however, one should know something about the rate of synthesis and elimination (or decay) of the hydrolytic enzymes mentioned.

The decrease in cytochrome oxidase activity in the whole kidney homogenate 12 and 18 hours[4] after injection of egg white (Table IV) confirms similar observations by Kretchmer and Dickerman (23). However, the decrease reported by these investigators was several times greater than the slight decrease of about 7 per cent found in the present experiments. Kretchmer and Dickerman (23) also reported marked changes of cytochrome oxidase activity in isolated fractions of the kidney homogenate after treatment with egg white. No striking changes were observed in the present experiments (Table I). These may not have been completely conclusive because of low recoveries of cytochrome oxidase (93 to 94 per cent in the experiment shown in Table I, 85 to 90 per cent in other experiments not recorded here). Since the recoveries were similar from the fractions of the treated and of the untreated animals, the comparison between both may still have been valid. Kretchmer and Dickerman (23) assumed that there were about equal amounts of droplets and mitochondria present in their mitochondrial fractions. It can be estimated, however, from the specific enzymatic activities of hydrolytic enzymes in purified droplets and in the original homogenate (reference 1, Table I), that the kidney contains not more than 5 per cent droplets. It may be noted in this connection that the crude fractions NDrI, DrII, and DrIII, described above, all contained an excess of mitochondria over droplets as indicated by the high cytochrome oxidase content of these fractions (Table I). For reasons discussed previously (1), it was suggested that the mitochondria are not the precursors of the droplets, as postulated by other investigators. The results of the present experiments do not require a modification of this view. This does not exclude the possibility that there may exist an indirect relationship between droplets and mitochondria, perhaps through hormonal effects.

It was suggested above that the injection of egg white induced the transformation of small droplets into large droplets and the concomitant release of a portion of the hydrolytic enzymes from the droplets into the surrounding cyto-

[4] Since Kretchmer and Dickerman (23) found the greatest decrease 12 hours after injection of egg white, kidney homogenates were tested 12 and 18 hours after injection. In both cases, an average decrease of about 7 per cent was observed.

plasm. As has been shown by de Duve *et al.* (21, 22) in the case of the hepatic lysosomes, the enzymes are active, *in vitro*, only after disintegration of the granules. A brief study is described in section *E* showing similar findings in the case of the kidney droplets. This transformation of the hydrolytic enzymes from an inactive to an active state is also likely to occur *in vivo* by the release of the enzymes from the droplets after treatment with egg white or other metabolic stimulation. In order to evaluate the functional state of these enzymes within the cells, it may therefore be more significant to determine the relative amounts of "free" and "bound" enzymes than their total amounts. Experiments are under way to test whether there exists a characteristic relationship between free and bound enzymes in different tissues.

It is interesting to compare the kidney droplets with the secretory granules[1] from other cells (3). It is known, for example, that the zymogen granules of the pancreas secrete their enzymes after metabolic stimulation, and that they contain proteases, nucleases, phosphatases (ATPase) and other hydrolytic enzymes. Whereas the hydrolytic enzymes of the zymogen granules of the pancreas have their optimal activity at a neutral pH, those of the kidney droplets and of the hepatic lysosomes have their optimal activity at an acid pH. The secretory granules from exocrine or endocrine glands extrude their products into ducts or the blood stream. The enzymes released by the kidney or liver granules appear to be mainly required for the functioning of the same cells, although they may also spill over into the blood or into other tissues. In this connection, the interesting work by Stevens and Reid (24) should be cited. These investigators found a decrease of acid ribonuclease and desoxyribonuclease in the granular fractions of liver cell homogenates and an increase in the supernatant fluid accompanied by an increase of these enzymes in the total homogenate, after adrenalectomy and hypophysectomy. These effects are similar to those occurring in kidney cells after treatment with egg white and suggest that the latter may also take place under hormonal regulation. Experiments are planned to test this possibility.

Questions may be raised as to where the enzymes of the droplets are synthesized, what their function is, and where the droplets themselves originate. It was suggested (3) that the droplets are derived from the Golgi region of the cells where acid phosphatase activity has been demonstrated histochemically (25, 26). It should be mentioned, however, that the Golgi material from epididymis, isolated by Schneider and Kuff (27), although rich in acid phosphatase, differed in several other respects from the kidney droplets. The Golgi region is often considered to function in the secretion and segregation of materials (28). This possible role of the kidney droplets in the "secretion" of enzymes has been discussed above, and the segregation of egg white (15) and of another protein (17) by the droplets has been observed. Both functions may be related, and the secretion of enzymes may be associated with the segregation or "active transport" of metabolites. The uptake of fluid and solid particles of colloidal size (so called pinocytosis and athrocytosis) has been described for the droplets of kidney cells (6), and for droplets in tissue culture cells (29, 30). De Duve *et al.* (2) have emphasized intracellular digestion ("lysosomes") as another possible function. Much more has to be learned about these processes before the function of the droplets and of their enzymes can be better understood.

SUMMARY

1. Kidney homogenates from rats injected with egg white and from control rats were fractionated simultaneously into six fractions and the content of acid phosphatase, ribonuclease, desoxyribonuclease, cathepsin, and β-glucuronidase in corresponding fractions from treated and untreated animals was compared. These observations were correlated with the amount of dark brown bottom sediments in fractions NDrI, DrII, and DrIII, and with the number of droplets in fraction NDrI.

2. It was found that after injection of egg white the amount of small droplets decreased as indicated by the decrease of the dark brown bottom layer in the sediment of fraction DrIII and by the concomitant decrease of hydrolytic enzymes in the same fraction, and that the number of large droplets increased as indicated by the increase of brown sediment in fraction NDrI and the increase in the number of droplets counted in a bacterial counting chamber in the same fraction. It was concluded that the treatment with egg white induced the transformation of small droplets into large droplets.

3. The decrease of hydrolytic enzymes in the fractions containing the small droplets was accompanied by a marked increase of these enzymes in the supernatant fluid. The enzyme content of fraction NDrI was not increased after treatment, although it contained greatly increased numbers of large droplets. Counting of the droplets in this fraction showed decreased enzymatic activity of the average large droplet after treatment with egg white. It was suggested that during the transformation of small into large droplets, a portion of the hydrolytic enzymes was released into the surrounding cytoplasm, and that this was partly responsible for the increased enzyme content of the supernatant fluid after fractionation of the kidney homogenate. In contrast to the four other hydrolytic enzymes, β-glucuronidase was not increased in the supernatant fluid.

4. Eighteen hours after intraperitoneal injection of egg white, the specific enzymatic activities of kidney homogenates showed a 25 to 35 per cent increase for cathepsin, ribonuclease, and desoxyribonuclease, no change for acid phosphatase and β-glucuronidase, and approximately a 7 per cent decrease for cytochrome oxidase. The increase of cathepsin, ribonuclease, and desoxyribonuclease in the total homogenate was interpreted as an indication of the formation of new enzymes, and it was suggested that this partly accounted for the increase of these enzymes in the supernatant fluid.

5. The activation of the enzymes by osmotic effects was investigated *in vitro* by incubation of droplet fractions in the presence of different concentrations of sucrose.

The author wishes to thank Miss Jenny Dinerstein for valuable technical assistance.

BIBLIOGRAPHY

1. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 513.
2. de Duve, C., Pressmann, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, 1955, **60,** 604.
3. Straus, W., *J. Biol. Chem.*, 1954, **207,** 745.
4. Terbruggen, A., *Beitr. path. Anat. u. allg. Path.* (Ziegler's), 1931, **86,** 235.
5. Laas, E., *Virchows Arch. path. Anat.*, 1932, **286,** 427.
6. Gerard, P., and Cordier, R., *Biol. Rev.*, 1934, **9,** 110.
7. Randerath, E., *Ergebn. allg. Path. u. path. Anat.* (Lubarsch and Ostertag's), 1937, **32,** 91.
8. Smetana, H., and Johnson, F. R., *Am. J. Path.*, 1942, **18,** 1029.
9. Smetana, H., *Am. J. Path.*, 1947, **23,** 255.
10. Oliver, J., *J. Mt. Sinai Hosp.*, 1948, **15,** 175.
11. Oliver, J., MacDowell, M., and Lee, Y. C., *J. Exp. Med.*, 1954, **99,** 589.
12. Zollinger, H. U., *Schweiz. Z. allg. Path. u. Bakt.*, 1950, **13,** 147.
13. Rather, L. J., *J. Exp. Med.*, 1948, **87,** 163.
14. Rather, L. J., *Medicine*, 1952, **31,** 357.
15. Straus, W., and Oliver, J., *J. Exp. Med.*, 1955, **102,** 1.
16. Mayersbach, H., and Pearse, A. G. E., *Brit. J. Exp. Path.*, 1956, **37,** 81.
17. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1037.
18. Hogeboom, G. H., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, **172,** 619.
19. Wattiaux, R., and de Duve, C., *Biochem. J.*, 1956, **63,** 606.
20. Rhodin, J., Correlation of Ultrastructural Organisation and Function in Normal and Experimentally Changed Proximal Convoluted Tubule Cells of the Mouse Kidney, Karolinska Institutet, Stockholm, Aktiebolaget Godvil, 1954.
21. Appelmans, F., and de Duve, C., *Biochem. J.*, 1955, **59,** 426.
22. Gianetto, R., and de Duve, C., *Biochem. J.*, 1955, **59,** 433.
23. Kretchmer, N., and Dickerman, H. W., *J. Exp. Med.*, 1954, **99,** 629.
24. Stevens, B. M., and Reid, E., *Biochem. J.*, 1956, **64,** 735.
25. Deane, H. W., and Dempsey, E. W., *Anat. Rec.*, 1945, **93,** 401.
26. Weiss, L. P., and Fawcett, D. W., *J. Histochem. and Cytochem.*, 1953, **1,** 47.
27. Schneider, W. C., and Kuff, E. L., *Am. J. Anat.*, 1954, **94,** 209.
28. Bourne, G. H., *in* Cytology and Cell Physiology, London, Oxford University Press, 1951.
29. Gey, G. O., Shapras, P., and Borysko, E., *Ann. New York Acad. Sc.*, 1954, **58,** 1089.
30. Rose, G. G., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 697.