CELLULAR MECHANISMS OF PROTEIN METABOLISM IN THE NEPHRON*

VI. The Immunological Demonstration of Egg White in Droplets and Other Cellular Fractions of the Rat Kidney after Intraperitoneal Injection

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In previous reports (1–5) the changes which are observed in the cells of the proximal convolution of the rat after the intraperitoneal injection of egg white and certain other proteins have been studied by histological, histochemical, biochemical, and enzymatic procedures. During the first 18 to 36 hours following the injection, cytological alterations were noted that have been interpreted as indicating an absorption of the protein by the cells of the proximal convolution of the nephron. Two aspects of this process could be distinguished; in an early phase, the absorbed protein was diffusely distributed throughout the protoplasm of the renal cell while at a later period droplets appeared.

It was found possible to isolate these droplets from homogenates of kidney tissue in relatively high purity but small yield, and biochemical analyses of them showed that they contained phospholipid and pentosenucleic acid in roughly similar concentration to that which occurs in the mitochondria (6). The droplets, both *in situ* (1) and when isolated (6), were also found to react positively to Janus green, a currently accepted specific stain for mitochondria.

In the case of certain proteins it was clear that the droplets also contained the injected protein; after the administration of hemoglobin their color indicated its presence and, as they later disintegrated, free Fe remained as further evidence. The droplets that formed after the injection of egg white, a mixture which contains a large proportion of the glycoprotein ovomucoid, were intensely positive to the periodic acid–Schiff (PAS) reaction,[1] which does not occur in

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[1] As shown in our Plate 35 (2) and in Davies' (7) illustrations (Plate 3) of the "spontaneous droplets" observed under normal and pathological conditions, PAS positivity in varying degree is characteristic of all these objects. In our experience none approach in the intensity of their reaction that which can be obtained by the administration of egg white. As Davies

the mitochondria (2). Droplets forming after the injection of all the proteins tested showed histochemically a high concentration of NH_2 bodies typical of protein whereas the mitochondria did not (2).

For these reasons it has been concluded that the droplets, containing absorbed protein and mitochondrial substances, represent *one* cytological aspect of the absorption of protein by the renal cells and it has been suggested that they act by means of mitochondrial enzymes which have been demonstrated within them (4, 5) as an accessory mechanism in the intracellular metabolism of the absorbed material.

The present report describes a further test of this hypothesis by examining with immunological methods the egg white content of pure suspensions of the droplets and other fractions of homogenates of the kidney tissues.

Materials and Methods

Male rats of the Sherman strain, 350 to 500 gm. in weight, were injected intraperitoneally with a mixture of 7 ml. of egg white and 7 ml. of saline. The homogenation and isolation of the droplets were carried out as described previously (6). The other fractions in the homogenate of the cortex of the kidney were isolated according to the method described by Hogeboom, Schneider and Palade (8).

Serological Estimation of Egg White.—As the samples of purified droplets are very small, the yield from the kidneys of 12 rats being in the order of 1 to 3 mg., it was found necessary to estimate the egg white content of the extracts by determination of dilution titers; *i.e.*, the highest dilution at which a positive reaction (precipitate) was obtained with a rabbit anti-egg white serum. Simultaneously in each experiment the same determination was made with a pure egg white solution of known N content and this was used as a standard to estimate the value of the analogous highest but still positive dilution of the unknown preparation. Each test consisted of a preliminary determination using dilutions of 1, 2, 4, 8 · · · times, and a final determination in which the highest dilution still reacting positively was further diluted to 8/10, 7/10, 6/10, 5/10, 4/10, and 3/10. All dilutions were made with M/30 phosphate buffer, pH 8.2, containing 0.7 per cent NaCl and 0.25 per cent phenol. The same mixture was also employed for the extraction of the egg white from the cell material. The extracts had to be clear for the serological reactions. This was usually the case after the extraction of the sediments with the salt mixture and subsequent high speed centrifugation, and what slight cloudiness remained in some samples became invisible at the high dilution to which they were brought for the serological reaction.

Capillary tubes were filled successively with 0.01 ml. of anti-egg white serum and 0.01 ml. of the test solution with the aid of a capillary pipette. The tubes were incubated for 1 hour at 37°C. and kept in the refrigerator for 4 days. The serological precipitates which settled on an air bubble at the bottom of the capillary tubes were observed each day through a magnifying glass but only the final readings after 4 days were used.

When the accuracy of the method was tested with pure egg white solutions of different

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suggests, the universality of PAS positivity is probably due to absorption of plasma glycoproteins from the tubule fluid. If any protein passes through the glomerular membrane the tubule fluid must contain these smallest of the plasma proteins and a consideration of the magnitude of the daily reabsorption of glomerular filtrate will indicate the importance of them in the production of PAS positivity in absorption droplets even though their concentration in plasma is low.

concentrations and known N content as "unknowns," errors in the range of 2 to 5 per cent were found. The validity of the method as used in the experiment, however, required that the kidney extracts not influence the serological reaction of egg white with its anti-serum. This was tested by comparing dilution titers of pure egg white solution with others to which kidney extract in a concentration of 0.1 to 0.3 mg. N/ml. had been added. No significant effect from the added kidney extract was observed at the high dilutions at which the serological reactions took place.

Reliable results in the analysis of the various cell fractions could only be expected if the egg white were completely extracted from the material. Experiments indicated that after 3 repeated extractions of the cell sediments with phosphate buffer–NaCl mixture no further egg white was detected in subsequent extracts.

As a general test of the procedure, the egg white content of a dry powder prepared from cortical tissue of the kidney from a rat injected with egg white was analyzed repeatedly.

TABLE I

Egg White Content of Renal Cortex at Intervals after Injection

Time After injection	$\frac{\text{Egg white N}}{\text{Total N}} \times 100$	
	Experiment 1	Experiment 2
hrs.		
3	0.2	
6	0.4	
9		0.6
13		0.8
18	1.5	1.4
24		1.3
30		0.7
40	0.1	

The results fell within 2 per cent of the same figure, 1.8 per cent egg white N per total N. In general, however, no accuracy greater than 10 per cent has been assumed for our data because of the many unknown factors inherent in serological reactions.

EXPERIMENTAL

Egg White Content of Renal Cortex at Intervals after the Injection

Rats were injected intraperitoneally with egg white and sacrificed at different intervals. The egg white content of the cortical tissue was estimated as already described.

As can be seen from Table I, the egg white content of the cortical tissue was highest at 18 to 24 hours after the injection. This agrees with histological observations indicating that the greatest numbers of droplets are visible *in situ* at this period (1). Antibody formation may have influenced the serological estimations in the later intervals, but the observed decrease in egg white content is in agreement with histological observation that the droplets decrease in number with time.

In the experiments that follow, the kidneys were removed approximately 18 hours after injection.

Distribution of Egg White in Cell Fractions of Kidney Cortex

Two experiments were made to determine, as far as possible, the distribution of egg white in nuclei, droplets, mitochondria, "microsomes," and supernatant fluid of the homogenate of the cortex of the kidney 18 hours after intraperitoneal injection. Throughout these and all the other experiments repeated microscopic examination was used to observe the particulate content of the suspensions. In the first experiment (Table II), the fractions were separated by a single sedimentation in the centrifuge; in the second experiment, the sediments were resuspended in 0.88 M sucrose solution and sedimented a second time for purification.

TABLE II

Distribution of Egg White in Cell Fractions of Renal Cortex

	Total N	Egg white N	$\frac{\text{Egg white N}}{\text{Total N}} \times 100$	$\frac{\text{Egg white N}}{\text{Total egg white N}} \times 100$
Experiment 1				
	mg.	*mg.*		
Residue, not homogenized	51.0	0.85	1.7	
Nuclei, blood, and renal cells. (droplets)	12.2	0.26	2.1	7.7
Droplets, nuclei. (cells, mitochondria)	17.4	0.77	4.4	22.7
Large mitochondria	21.5	0.51	2.4	15.0
Small mitochondria. (microsomes)	24.0	0.36	1.5	10.6
Microsomes	10.4	0.11	1.1	3.2
Supernatant fluid. (microsomes)	93.0	1.38	1.5	40.7
Experiment 2				
Residue, not homogenized	33.3	0.60	1.8	
Droplets, nuclei. (large mitochondria)	9.7	0.46	4.7	20.8
Mitochondria	33.9	0.29	0.9	13.1
Microsomes	1.2	0.05	0.4	2.3
Supernatant fluid. (microsomes)	109.0	1.41	1.3	63.8

In both instances the separation of the cell particulates was incomplete, the nuclear and droplet fractions in particular containing bodies characteristic of other fractions. In the tables the nature of these contaminating particles has been indicated in parentheses. The fourth column of the tables refers the egg white N of each fraction to the total N of the same fraction, whereas the fifth column refers the egg white N of each fraction to the total egg white N of all fractions combined. The residue after homogenization was not included in the calculation. As has been noted, the use of an all glass homogenizer may destroy droplets; the tissue residue was therefore considerable.

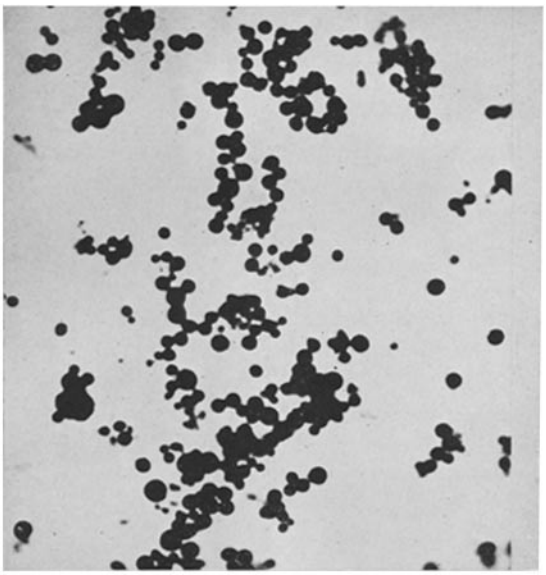

FIG. 1. A thin smear of a purified droplet suspension fixed with OsO_4 and stained with gentian violet. × 400.

TABLE III

Egg White Content of Highly Purified Droplets

No. of experiment	$\frac{\text{Egg white N}}{\text{Total N}} \times 100$				
	Droplets	Total kidney	Mitochondria	Microsomes	Supernatant fluid
1	4.5	1.0	1.2	1.1	1.2
2	5.8	1.5			
3	5.4	1.3			
4	5.5	1.2			
5	6.0	1.5			
6	6.8	1.4			
7	8.2	—	1.6	1.5	—

Egg White Content of Highly Purified Droplets 18 Hours after Injection

Table III shows the serological estimation of egg white in suspensions of highly purified droplets made 18 hours after injection of the egg white.

The droplets were isolated by repeated differential centrifugation and filtration through cotton (6) and on microscopic examination appeared to be composed of droplets only (Fig. 1). The material of Experiment 1 was purified by repeated differential centrifugation without filtration and was less pure.

For comparison, the egg white content of the total cortical tissue, and in some experiments, of the mitochondria, "microsomes" and supernatant fluid was also determined. The egg white N of each fraction was referred to the total N of each fraction.

As can be seen from Table III, the droplets were found to contain an average of 6.0 per cent egg white N per total N, the other cell fractions and the total cortical tissue only 1 to 1.5 per cent.

Degree of Fixation of Egg White within Droplets

An experiment was made to determine whether egg white is firmly bound in the droplets. Three 1 ml. samples of the same droplet suspension were shaken vigorously with 9 ml. of 30 per cent sucrose solution, the first sample once, the second twice, and the third three times and sedimented after each shaking by high speed centrifugation. The egg white content of the droplets after one, two, and three shakings was found to be 6.2, 6.4, and 5.1 per cent egg white N per total N, respectively. These results indicate that the egg white is relatively firmly bound in the droplets. The clear supernatant fluids of the shaken and resedimented droplets were found to contain considerable amounts of egg white. This must have resulted from the disintegration of some droplets as a result of the vigorous shaking, and a release of egg white into the suspending solution.

DISCUSSION

A critical consideration of our findings and conclusions should deal with the following points:—

The method of cell homogenation involved the disruption of cell integrity and conceivably may have allowed a redistribution of the substances under examination by diffusion and adsorption. This general criticism of the technique has been extensively discussed by others in recent years and the findings of our experiments add nothing new to its consideration. They obviously are subject to the general limitations of the method.

The fractions of the homogenate were not strictly pure, if indeed such is ever the case by this technique. In our experiments purity of fractionation could only be claimed for the highly purified suspension of droplets prepared by repeated differential centrifugation and filtration through cotton. Moreover, other difficulties arose in our fractionating; for example, our estimation of the egg white content of the mitochondria must have been too low, as we have shown that the phenomenon of mitochondrion-droplet transformation is limited to the proximal convolution (1) and our preparation contained mitochondria from all the cortical cells. The greater bulk was derived from the area of cellular change, but the mitochondria were certainly somewhat diluted by similar extraneous elements from tubules not concerned in the process.

The immunological procedure used for the estimation of the egg white content of the fractions was not so accurate as others in which the N of the serological precipitate is determined. To use such a procedure was impossible in the case of the suspension of purified droplets owing to the small amount of material. Although larger amounts of the other fractions were available it seemed best to use the same method throughout. No definitive quantitative significance is therefore claimed for the values obtained, but it is believed that the data are valid for purposes of comparison of similarly tested fractions. The simple qualitative demonstration, in fact, of the presence of egg white proteins in the droplets in considerable amount is of considerable value, as will be emphasized later.

A critical evaluation of the uncertainties noted above is facilitated if they are considered in the light of our previous histochemical and cytological studies.

For example, in the present experiments the amount of egg white in the cortical tissue of the kidney was found by a specific immunological method to increase to a maximum at 18 to 24 hours and then decrease. This observation correlates with the known course of its elimination in the urine and with the histological evidence of its presence in the cells of the proximal convolution.

The partition of the egg white in fractions of the renal cells at 18 hours (Table II) showed 40 to 60 per cent of the total in the supernatant fluid, 15 per cent in the fraction which was chiefly mitochondrial, and 20 per cent in the fraction of which the droplets were the predominant component, and as the examination of purified preparations showed (Table III), in a concentration 4 to 6 times that of any other cell particulate. This is a pattern of distribution that agrees with previous histological, histochemical, and biochemical findings and with the hypothesis outlined in earlier investigations (1, 2). According to this hypothesis, the protein, egg white, is absorbed by the renal cell and diffusely infiltrates its cytoplasm. A high concentration of "free" egg white is therefore to be expected and is in fact found in the supernatant of the cellular homogenate.

According to the hypothesis mentioned, droplets, which have been shown to contain phospholipid and pentosenucleic acid and to be Janus green–positive, are formed by a combination of the intracellular egg white protein with the mitochondria. This interpretation does not exclude the possibility that other as yet undetermined cytoplasmic substances may also be present; for example, enzymes have been found that are not strictly localized to the mitochondria (6). Our present observations indicate that 6 per cent at least of the droplet protein is immunologically egg white, a concentration 4 times that found in any other cell constituent. As the hypothesis further suggests, the association of absorbed egg white protein with the mitochondria is demonstrable before the droplets are completely formed as individual entities, a lesser but still considerable amount of egg white being found fixed to the mitochondrial fraction.

Further clarification and support of the hypothesis result when the findings of our previous histochemical studies and these immunological findings are compared with those of Rhodin (9), who has recently examined by high resolution electron microscopy the changes that occur in the renal epithelium of the proximal convolution of the mouse after the injection of egg white.

In Rhodin's photographs of the mitochondria of the normal renal epithelial cell the characteristics of these cellular organelles, their external double membrane, the multiple internal membranes, and the "opaque spots" permit an absolute identification of them which is not possible by conventional staining methods and light microscopy. After the injection of egg white he observed, as had we by means of light microscopy (1), a marked decrease in the number of mitochondria. According to his description there presumably occurred in the *first stage* confluences of several mitochondria, which coincidentally became surrounded by a "finely grainy substance" that, with the mitochondria, formed large granules. Within these granules the mitochondria could still be perceived, owing to the fact that their ultrastructure was retained. In the *second stage*, the characteristic mitochondrial structure was lost and the cell became filled with "finely grainy granules" 2 to 4 μ in size. The number of the mitochondria was now less than normal and the area of the mitochondria was markedly decreased.

In the light of Rhodin's observations our previous biochemical findings of phospholipid in high concentration (6) and our histological demonstration of Janus green positivity (1) become understandable, for in the electron photographs the mitochondrial element of the droplets could be seen. These objects cannot therefore have been what Davies has previously supposed, simple droplets of absorbed protein (7).

The nature of the "finely grainy substance" which, according to Rhodin, surrounded the mitochondria transforming them to granule-droplets 4 μ in diameter was not disclosed by the electron microphotography which showed only an accumulation of a granular material not visible in the cells prior to injection of the protein. To suppose that it may have been some component derived from the mitochondrion itself or from the original cell cytoplasm as an effect, possibly "osmotic," of protein in the lumen of the tubule, seems an unwarranted assumption because we have shown that the swollen mitochondria and the fully formed droplets contain substances which are not present in the mitochondria but which give both to it and to the "grainy" element the histochemical and specific immunological reactions of the egg white proteins which were injected and which were passing through the renal tubule.

Since the original mitochondria are entirely negative to the PAS reaction (7, 2), and certainly contain no egg white, it would seem reasonable to suppose that the "finely grainy substance" which, in the electron microphotograph, surrounds and deforms the mitochondria, fusing ultimately with their substance

to form the droplet, contains the glycoprotein of the injected egg white. If this be so, the absorption of protein by the renal cell and certain aspects of the mechanism concerned are demonstrable not only by histochemical, biochemical, and immunological analysis but can be visually observed by high resolution electron microscopy.

SUMMARY

Immunological analysis with a specific antiserum showed an increasing concentration of egg white proteins in the cortex of the kidney of the rat up to 18 hours after their intraperitoneal injection, and a fall in the following 40 hours.

Homogenates of the cortical tissue prepared at 18 hours, the period of maximum droplet development, showed a large amount of the egg white proteins in the supernatant, considerable amounts in the fraction which contained the mitochondria, but the highest concentration, 6 times that of any other cell fraction, in the highly purified preparation of droplets.

The relation of these findings to the morphological and histochemical evidence concerning the constitution of the droplets is discussed and the conclusion drawn that absorbed egg white proteins and mitochondrial substances are important elements in their formation.

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