L. LYOCHROMES IN THE KIDNEY. WITH A NOTE ON THE QUANTITATIVE ESTIMATION OF LYOCHROMES

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ELLINGER & HIRT [1929, 1, 2, 3; 1930, 1, 2; 1931] demonstrated the presence of a yellowish-green fluorescent substance in the kidney during their investigation of the function of the frog's kidney by means of intravital microscopy. This substance was extracted and shown to have the same chemical properties as the lyochromes isolated from whey by Ellinger & Koscharka [1933, 1, 2, 3; 1934] and simultaneously and independently by Kuhn *et al.* [1933, 1, 2, 3] from egg-white. In spite of many investigations carried out on these pigments nothing is known about the localization of the lyochromes in the different parts of the kidney. The following experiments were therefore made by the method of intravital microscopy to investigate their distribution in the kidneys of normal frogs, normal rats and rats fed on a lactoflavin-deficient diet as well as in the kidneys of these animals after injection of lactoflavin. At the same time the methods used for the quantitative estimation of the lyochrome content of tissues were investigated.

EXPERIMENTAL

The distribution of lyochromes in the frog's kidney

The frogs used were large specimens of *Rana esculenta* obtained from Hungary, weighing 70–100 g. They were kept in a dark and cool basement in a tank where they could sit either in water or on a dry board. For periods varying up to six months they received no food. Before the experiments the frogs were prepared as described by Ellinger & Hirt [1929, 1]. They were narcotized percutaneously with urethane; the abdominal skin was cut through, the abdominal vein ligatured and divided, the abdominal cavity opened and the kidney exposed to the objective of the intravital microscope. Fluorescent substances of two different types were seen.

(1) A yellowish-green fluorescent substance was present in low concentration in the capsules of active glomeruli and in much higher concentration in the lumina of all the corresponding tubules and also in the epithelium of the proximal tubules. The substance was absent from the lumina of all tubules connected with inactive glomeruli (cf. Figs. 1, 2).

(2) In addition, a substance with a more yellowish fluorescence was observed only in the epithelium of the proximal tubules; it was present as small granules either distributed uniformly in the whole cytoplasm or, more often, accumulated in that portion of the cells which bordered on the lumina. The lymph spaces between the cells showed no fluorescence. The yellow fluorescent granules could be observed more easily in the proximal tubules connected with inactive glomeruli which were free from the greenish fluorescent lyochromes. The epithelial cells of the glomerular capsules and of the distal and collecting tubules contained no fluorescent material (cf. Figs. 2, 3).

After the death of the cells the yellowish-green fluorescent substance spread diffusely over the whole tissue of the kidney while the yellow fluorescent substance remained confined to the epithelial cells of the proximal tubules. The above appearances were noted in frogs freshly caught as well as in those which were kept for many months without food.

In order to examine the behaviour of the frog's kidney towards higher concentrations of lactoflavin in the blood, frogs which had been examined under the microscope as described above received injections of 20–80 γ lactoflavin dissolved in Ringer's solution into the lymph sac of the leg. A few minutes later the blood in the renal arteries became fluorescent, and a fluorescence of about equal intensity was almost simultaneously observed in the capsules of the active glomeruli; a little later there was a much brighter fluorescence in the lumina and epithelia of the proximal tubules and in the lumina of the lower urinary tract. The lumina of the proximal and distal tubules were much brighter than those of the glomeruli and tubules had been before the injection of lactoflavin. This increased fluorescence disappeared 2–5 hr. after the injection, the time depending on the amount of lactoflavin injected (cf. Fig. 4).

The distribution of lyochromes in the rat's kidney

The rats used in the experiments were from the stock of the Lister Institute and of about 200–250 g. weight. They were narcotized with 1 ml. of 0.03 % solution of chloralhydrate per 100 g., injected intraperitoneally. A small incision was made through the skin of the back, the fascia and the muscles. The kidney was brought to the surface through the slit thus made by means of slight pressure on the ventral side and fixed on a small metal plate containing an opening for the hilus. The plate was fixed to the stage of the intravital microscope in order to avoid any movement and the kidney was decapsulated. During examination the rat was fixed on a heated stage and the surface of the exposed kidney was irrigated with Ringer's solution heated to 40°. As only proximal tubules are present on the surface of the rat's kidney it was necessary to slice a small section from the top of the kidney [*vide* Ellinger, 1934] to expose the glomeruli and the distal tubules.

In the rat's kidney, as in the frog's, both greenish and yellowish fluorescent substances were found. The former was present in low concentration in the glomerular capsule and in higher concentration in the epithelial cells of the proximal tubules and in some of the lumina of these and of the distal tubules; most of the lumina, however, were dark. All the proximal tubules contained yellowish fluorescent granules distributed throughout the cells but concentrated more thickly round their edges. In the tubules which contained the greenish fluorescent substance, the yellowish fluorescence was masked to a great extent. The epithelial cells of the glomerular capsules and of the distal tubules were free from fluorescent material (cf. Fig. 5).

In order to observe the effect of injecting lactoflavin, the top portion of the kidney was removed at a special operation carried out a few days previously under ether narcosis. At the time of the experiment the rat was narcotized with chloralhydrate as described above and fixed on the microscope stage; the kidney was brought to the surface. 1 mg. of lactoflavin, dissolved in 2 ml. of Ringer's solution, was injected into the peritoneal cavity. The capsules of the active glomeruli immediately became brighter; a few seconds later a bright yellowish-green fluorescence was emitted by the lumina of the proximal tubules and almost simultaneously by the epithelial cells of these tubules and by the

lumina of the distal tubules. This increase in intensity of fluorescence was observed only in urinary systems which had shown some greenish fluorescence before the lactoflavin was injected. About 2-2½ hr. after the injection the intensity of the green fluorescence began to decrease and about 1 hr. later the picture was the same as before the injection of lactoflavin.

The distribution of lyochromes was further studied in the kidneys of rats which had received a diet deficient in the whole vitamin B₂ complex, including lactoflavin, for about 6 weeks and which showed symptoms of the deficiency. The feeding of the rats was carried out by Miss C. E. Edgar to whom I am much indebted. The kidneys of these animals, examined with the intravital microscope, showed a diminished but still considerable fluorescence in the same areas of the kidney as in normally fed rats. The concentration, both of the greenish fluorescent pigment in the lumina of the urinary units and of the yellowish fluorescent material in the epithelial cells of the proximal tubules was reduced in comparison with normal control rats, the latter pigment, however, to a less extent than the former.

DISCUSSION

The microscopic examination of the distribution of lyochromes in the kidney of the frog and rat showed these pigments to be present in at least two different states. One pigment emitting a yellowish-green fluorescence was found in the lumina of the urinary tubules belonging to active glomeruli, in increasing concentration from the glomerular capsule to the ureter; it was also present in the epithelial cells of the proximal tubules of these active units, where it stained the cytoplasm diffusely. The yellowish-green fluorescent material present in the kidneys of frogs and rats is probably free lactoflavin, which is excreted through the kidney and partly reabsorbed in the proximal tubules. When extracted it had the properties of lactoflavin [Ellinger & Koschara, 1933, 1, 2, 3]. The experiments in which lactoflavin was injected into frogs and rats confirmed this view, since after the injection the yellowish-green fluorescence of the lumina was greatly increased. The mechanism of the excretion of lactoflavin is similar to that of fluorescein, described for the frog by Ellinger & Hirt [1929, 3; 1930, 1] and for the rat by Ellinger [1938]. Lactoflavin, like fluorescein, is found spread over the whole tissue after the death of the cell. This was described by Ellinger & Hirt [1930, 2] as being due to a fixation determined by the static potentials of the living cell.

It is probable that the granules with yellowish fluorescence seen in the epithelial cells of the proximal tubules also consisted of lyochromes, since they were reduced in amount in kidneys of rats which had been deprived of lactoflavin in their diet. The distribution of these granules remained unchanged after the death of the cell in a manner similar to that shown by acriflavin [*vide* Ellinger & Hirt, 1930, 2]. These granules are probably fixed in the protoplasm by some chemical or physico-chemical mechanism. This yellowish fluorescent pigment is not apparently a lactoflavin-phosphoric acid-protein compound such as the yellow enzyme of Warburg & Christian [1932, 1, 2], since this enzyme is not fluorescent and if present in the kidney would not be demonstrated by the above method.

The epithelial cells of only the proximal tubules contained green fluorescent lyochromes; the pigments were probably reabsorbed from the lumen, since, when lactoflavin was injected, the green fluorescent lyochromes entered the epithelium from the lumen. No evidence was obtained as to the way in which the yellow fluorescent material entered the epithelial cells.

Frogs kept without any food for months continued to excrete lyochromes in the urine and also showed flavins in granules in the epithelium of the proximal tubules. The concentration of flavins did not appear to diminish with this long period of starvation, which suggests that the frog may be able to synthesize lyochromes. Young rats, on the other hand, which had been deprived of lactoflavin in their diet for about 6 weeks showed a great reduction in the amount of free lyochromes in the lumina of the urinary tubules and a smaller reduction in granules visible in the epithelium. But even those rats which bore the signs of severe vitamin B₂ deficiency still showed a small but continuous excretion of lyochromes. Kuhn *et al.* [1935] also described a reduced but still considerable amount of lactoflavin in the organs of vitamin B₂-deficient rats.

Attempted quantitative estimation of lyochromes

Attempts were made to estimate quantitatively the lyochromes present in the kidneys of normal rats and of rats deprived of the vitamin B₂ complex. The kidneys were minced, ground with sand in a mortar and extracted with water, dilute alcohol or dilute acetone. After repeated extractions the extracted material still showed a yellowish fluorescence under the intravital microscope. Extraction with these solvents was still incomplete after the tissues had previously been boiled in dilute HCl.

Liver, yeast and wheat germ were treated similarly; these also could not be freed completely from the fluorescent material by extraction with the above solvents. Attempts at quantitative estimation of the lyochromes contained in the kidney were therefore abandoned.

The methods which have been used by other workers for the concentration and purification of lyochromes obtained from tissues were also studied. In most of these the lyochromes after extraction were concentrated by adsorption on fuller's earth and subsequent elution with a pyridine-water-acetic acid mixture.

A definite amount of lactoflavin was added to an aqueous kidney extract which was treated with different preparations of fuller's earth. The adsorption was sometimes incomplete and sometimes complete; the more complete the adsorption, the more incomplete was the elution. After complete adsorption the adsorbate which had been treated 15 to 20 times with the above elution-mixture still yielded some fluorescent material on subsequent elution. The eluate obtained from tissue extracts always contained pigments other than flavins which prevented colorimetric or fluorimetric estimation of the lyochromes. The complete destruction of these coloured impurities by oxidation with potassium permanganate, as carried out successfully by Koschura [1934] with the eluates obtained from urine, was usually not possible in the eluates obtained from the animal and plant tissues examined.

Attempts to estimate the lyochrome content of the tissue extracts by measuring the intensity of ultraviolet absorption at wave-length 2650 Å. were without success. All the extracts showed a general absorption in the ultraviolet beginning at about 3500 Å., due to impurities which could not be destroyed without destruction of the lyochromes.

The concentration of lyochromes in the eluate has previously been estimated either by measurement of the intensity of the fluorescence of the lactoflavin contained in the eluate [*vide* v. Euler & Adler, 1934; Cohen, 1934; 1935, 1, 2; Josephi, 1934] or by colorimetric estimation of the lumiflavin content of a chloroform extract from the aqueous eluate after conversion of the lactoflavin into lumiflavin by exposure to light in alkaline solution [Warburg & Christian, 1933; Kuhn *et al.* 1934].

Attempts were also made at quantitative estimation of the lyochromes in the eluates by comparing the intensity of their fluorescent light with that of a solution of lactoflavin of known concentration. For this purpose solutions containing from 1 to 200 γ pure lactoflavin per ml. were compared. The differences in intensity of fluorescence excited by the light of a 10 amp. arc lamp passed through a filter UG 2, Schott, Jena, were measured in a Bausch and Lomb colorimeter used as a nephelometer. The readings bore no relation to the concentration of lactoflavin and were largely dependent on the particular conditions of lighting and the width of the layer of solution. Concentrations differing by as much as 10 times sometimes gave equal intensities of fluorescence.

The procedures used for the extraction, concentration and purification of lyochromes from animal and plant tissue were shown to involve considerable losses of material. The colorimetric method for the estimation of lactoflavin concentrations suggested by Warburg & Christian [1933] and used on a larger scale by Kuhn *et al.* [1934] is marred by heavy and inconstant losses, calculated by Kuhn and his co-workers to be between 13 and 58%.

The fluorimetric estimation of lyochrome concentration is also unreliable. In this method the intensity of the fluorescence is influenced by the neutral salt content of the solution and by the presence of coloured impurities, from which extracts of animal and plant tissues cannot be freed completely. Further, the above experiments with solutions of pure lactoflavin of different concentrations showed that it is generally impossible to estimate the amount of a fluorescent pigment even in a pure solution. It is only by chance that correct results can be obtained by this method; very dilute solutions give the best chances of success. Kuhn & Moruzzi [1934] used this method to investigate the dependence of lactoflavin fluorescence on small changes in pH. They showed that the intensity of fluorescence was proportional to the concentration of lactoflavin, but not to that of *NN'*-dimethylalloxazine. Kuhn *et al.* [1934] abandoned this method when examining unknown concentrations of flavins in plant tissues, in favour of the colorimetric estimation of lumiflavin, a method which is also inaccurate for other reasons. Fink & Hoerbuerger [1933, 1, 2; 1934; 1935] examined very carefully the accuracy of the fluorimetric estimation of porphyrin solutions of unknown concentration and showed that it was impossible to obtain reliable results.

The intensity of the fluorescence measured in a nephelometer is dependent on the amount of absorption as well as on the amount of emission. The relation between the intensity of the fluorescent light and the concentration of pigment is very complicated. The analogous problem of the variation of the intensity of scattered light with the concentration of sols was studied by Krishnamurti [1929]. Rinde [1934] showed that it was possible to find a formula for the variation of the intensity of Tyndall-light with the concentration of sols taking into account absorption as well as emission. This formula is in practice only valid when a constant and invariable source of light is available, a condition which cannot be maintained at present in fluorimetry [*vide* also Zerban & Sattler, 1937]. The source of ultraviolet light has to be of the highest possible intensity and of point form, which can be obtained only by using a carbon arc lamp. Only when the intensity of the exciting light is so great that it passes the layer of pigment solution without significant loss of intensity can the intensity of the emitted fluorescence be proportional to the concentration of the pigment. An approximation to the attainment of this condition could be secured by diminishing the pigment layer to a minimum; this, however, would limit the accuracy of the measurement.

It might be possible to use the intensity of fluorescence as a measure of the concentration of the fluorescent substance by diluting the solution of unknown concentration with water till the visible fluorescence just disappears [*vide* Whitnak *et al.* 1937]. If the smallest concentration of the fluorescent pigment which just gave a visible fluorescence and the amount of dilution were known the concentration could be estimated. But even so impurities and salts might influence the limiting value of the fluorescence.

Under these circumstances the most satisfactory method seems to be that of biological estimation, in which the growth-promoting power of the material containing lyochrome is compared quantitatively with that of pure lactoflavin. This method is laborious, and, like all biological methods, has a large margin of error. The chief difficulty is to provide a basal diet sufficiently free from lactoflavin. This method has been used by Copping [1936] and by Edgar *et al.* [1937]; Copping & Roscoe [1937] who used it for determining the flavin content of baker's yeast found at least 180 γ per g., an amount 5–10 times greater than that obtained by workers using the above chemical and physico-chemical methods [Warburg & Christian, 1933; Kuhn *et al.* 1934; v. Euler & Adler, 1934]. The figures for the lyochrome contents of various plant and animal tissues derived from the chemical or physico-chemical methods of estimation discussed above are, therefore, probably much too low.

SUMMARY

1. The distribution of the lyochromes in the kidneys of frogs and rats was studied by intravital microscopy. Two types of lyochromes were found: (a) a yellowish-green fluorescent pigment in the lumina and in the epithelial cells of the proximal tubules of active urinary units; these pigments are excreted and partly reabsorbed in the proximal tubules: (b) a yellowish fluorescent pigment distributed in granules in the epithelial cells of all proximal tubules whether belonging to active or inactive units.

Lactoflavin injected into animals was eliminated in the glomeruli, concentrated and partly reabsorbed in the proximal tubules and concentrated further in the lower parts of the urinary tract. Its behaviour was similar to that of fluorescein. The yellowish fluorescent lyochromes fixed in granules in the proximal tubules showed some similarity with acriflavin.

2. Frogs kept without any food for several months excreted lyochromes continuously in the urine and showed abundant yellowish fluorescent material in the epithelium of the proximal tubules. Rats deprived of lactoflavin in their diet excreted lyochromes in smaller amounts than normally fed rats and frogs. In such rats lyochrome granules were still present in the epithelium of the proximal tubules, but in smaller amounts than in normally fed rats.

3. The chemical and physico-chemical methods used for the quantitative estimation of lyochromes in plant and animal tissues have been discussed and the errors involved in them have been pointed out. The conclusion is reached that the figures obtained by these methods are much too low. The biological estimation of the lyochrome content is considered to be the most satisfactory method.

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DESCRIPTION OF PLATE VI

Figs. 1-5. Photographs of living kidneys. Special lenses for intravital microscopy and Schott light filters (BG 3, 3.5 mm. and GG 5, 1 mm.) were used in taking these photographs.

Fig. 1. Frog's kidney, ventral side; (a) glomerulus; (b) distal tubules. Greenish fluorescent lyochromes in the glomerular capsule in low concentration and in the distal tubules in higher concentration.

Fig. 2. Frog's kidney, dorsal side; proximal tubules: the bright ones in the centre (a) contain greenish fluorescent lyochromes in lumen and epithelium, the darker ones (b) only yellowish granules in the epithelium.

Fig. 3. Frog's kidney, dorsal side; one proximal tubule: yellowish fluorescent granules in the epithelial cells (a) cell borders, (b) dark.

Fig. 4. Frog's kidney, ventral side; glomerulus (a) and one proximal tubule 8 min. after the injection of 0.5 ml. lactoflavin sol. 1/12,000. Epithelium (b) and lumen (c) of the proximal tubules very bright.

Fig. 5. Rat's kidney; proximal tubules, yellowish fluorescent epithelia; (a) one greenish fluorescent tubule, (b) in the lower right corner.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.