Variations in Melanin and Riboflavin Content of Amphibian Liver under the Influence of Reserpine and Amphetamine

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Melanin, Riboflavin, Liver, Reserpine, Amphetamine Effects

By means of paper chromatography, *Lactobacillus casei* test and the staining method with the Feulgen reaction, the effects of reserpine and amphetamine on riboflavin and melanin were studied in the liver of *Triturus cristatus*. It was demonstrated that the concentrations of both melanin and riboflavin can be altered by the influence of these drugs. From the results obtained it is suggested that riboflavin shows a correlation with the density of melanin.

Introduction

In a recent series of experiments, in which we investigated the effects of reserpine and amphetamine on cellular substances other than catecholamines, we found that these two drugs alter the pteridine pattern and the Feulgen-stainability of the nuclear chromatin in the liver of *Triturus cristatus* [1]. The behavior of the animals, expressed in motility, was also modified by the two drugs in opposite ways.

Reserpine and chlorpromazine are known to produce a parkinsonian syndrome in man [2] and it was also found that long-term therapy with these drugs causes demelanization of the substantia nigra [3]. This demelanizing action seems paradoxical since reserpine and chlorpromazine, as well as other phenothiazines induce increased melanization of the skin of amphibians to varying degrees, which however parallelize their tranquilizing potency in man [4]. On the other hand amphetamine has been used for the alleviation of parkinsonian symptoms [5] and is generally known to reverse reserpine sedation [6]. Furthermore, the highest concentration of riboflavin in the brain has been found in the substantia nigra and basal ganglia [7] and it was shown that it can be lowered drastically by oxotremorine [8], a drug that produces a state very similar to Parkinsonism [9].

Since Barbeau has recently presented convincing arguments in favour of implicating the liver in the origin of enzymatic defects in Parkinsonism [10], we considered it of interest to present the second part of our investigation [1] concerning melanin and riboflavin now, in order to determine whether the amphibian liver melanocytes reacted in a way similar to substantia nigra neurons under the influence of drugs that affect the function of these neurons.

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Materials and Methods

Triturus cristatus adult males were used in this study. The methods employed have been described elsewhere [1]. Two groups of animals (of 10 each) were injected, under MS 222 (1 : 2500) narcosis, with 0.11 mg/5 g animal of reserpine (Serpasil, Ciba) and with 2.5 mg/5 g animal of D-amphetamine sulphate dissolved in amphibian Ringer (8 g NaCl, 420 mg KCl, and 250 mg $CaCl_2$ in 1000 ml distilled H_2O). The criterion for determining the amounts of reserpine and amphetamine to be injected into *Triturus* was the equivalence of doses which produce behavioral effects in man. A third group of 10 animals, free of drugs, was used as control. Animals from each group were sacrified at 4, 8 and 24 h after injection. The liver was removed and divided into two halves. One piece was freeze-dried and stored at −5 °C for the chemical determinations, the other piece was fixed for histological sections.

The extraction of pteridines and riboflavin from the liver was effected according to the method of Kokolis and Ziegler [11]. The identification of the different pteridines, as well as of tetrahydrobiopterin and riboflavin is described in *l. c.* [11]. The quantitative determination of riboflavin was done by the growth test with *Lactobacillus casei* [11, 12]. Paraffin sections of formalin fixed liver were stained with the Feulgen method for DNA, without further counterstaining as is described in *l. c.* [1]. Photomicrographs were taken with the Orthomat Camera fitted on the Leitz Ortholux microscope.

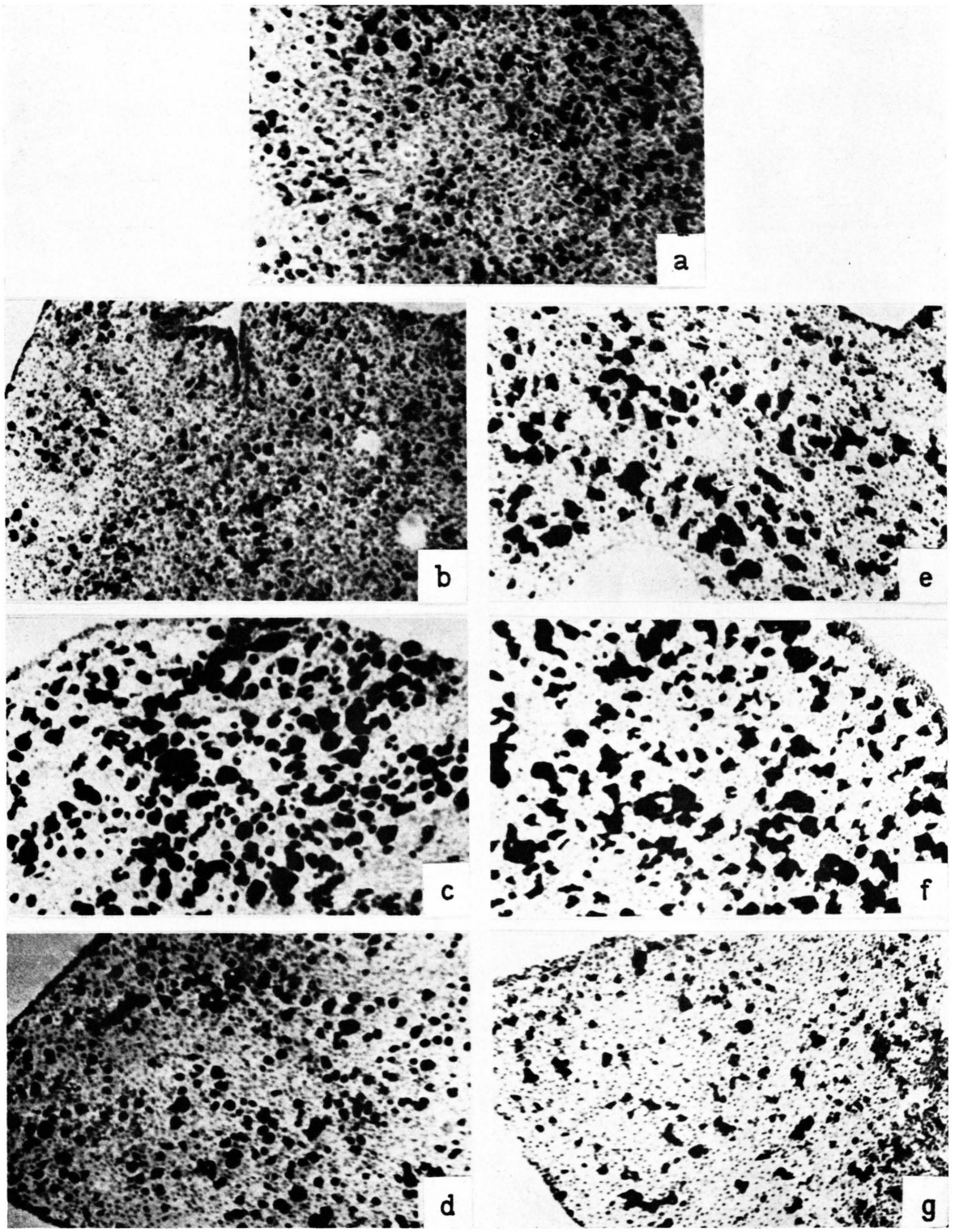

Fig. 1. Micrographs of *Triturus cristatus* liver. X 120. a. control, b. 4 hours after reserpine, c. 8 hours after reserpine, d. 24 hours after reserpine, e. 4 hours after amphetamine, f. 8 hours after amphetamine, and g. 24 hours after amphetamine.

Results

The melanocytes in the liver of control *Triturus cristatus* (Fig. 1 a *) form small clusters more or less evenly distributed throughout the tissue. The size of the clusters varies to some extent; the outlines are mostly rounded and occasionally irregular. By the staining method used, melanin retains its natural brownish-black color and only the nuclei stand out very clearly both in the melanocyte clusters and in the hepatic cells. The granules are densely packed. At 4 hours after reserpine (Fig. 1 b) the number of clusters is considerably smaller when compared to the control. The clusters have a uniformely small size and mostly rounded shape. The melanin granules seem fewer per unit area. In the livers of the 8-hour reserpinized animals we found an enormous increase in both the number and the size of the melanocyte clusters (Fig. 1 c). A great number of nuclei is evident in each cluster. The morphology of these nuclei is very divergent from that of the hepatocyte nuclei. The melanocytes are densely packed with granules and show smooth, rounded outlines. At 24 hours after reserpine (Fig. 1 d) the number and size of the melanocyte clusters in the liver are about the same as in the control.

After the administration of amphetamine we find a different sequence of melanin variations. At 4 hours after injection (Fig. 1 e) the melanocyte clusters are of a very large size if not more in number. They are characterized by a very irregular "dendritic" outline. The melanin granules are loosely packed in the cells. At 8 hours after amphetamine (Fig. 1 f) are of still larger size and are very numerous. Their outline is still branched and thorny. The liver of the animals at 24 hours (Fig. 1 g) contains very few clusters of melanosomes. Their number is much smaller than that of control animals and even smaller than that of the 4-hour reserpinized animals. Observation of these sections under high magnification revealed the presence of melanin-free or demelanized clusters of cells recognizable by the morphology of their nuclei and by the inclusion of very few scattered, single melanin granules.

The measurements of riboflavin content of the whole liver tissue at the different time intervals after reserpine and amphetamine are shown in Figs 2 and 3 respectively. We have included in the figures also the values of tetrahydrobiopterin and isoxanthopterin from the same experiments in order to be able to discuss our results with these previous findings [1].

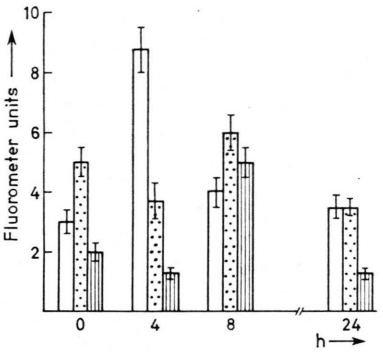

Fig. 2. Effect on pteridine and riboflavin concentration of reserpine. □: tetrahydrobiopterin; ⁝⁝: isoxanthopterin; ▥: riboflavin. Values refer to the mean ± standard error of the mean of three determinations.

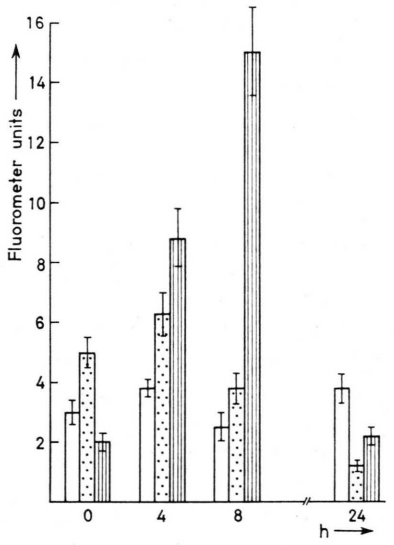

Fig. 3. Effect on pteridine and riboflavin concentration of amphetamine. □: tetrahydrobiopterin; ⁝⁝: isoxanthopterin; ▥: riboflavin. Values refer to the mean ± standard error of the mean of three determinations.

The administration of reserpine, after an initial reduction in the riboflavin content of the liver, induces a maximum in the value at 8 hours and a further drop at 24 hours. In contrast to the effect of reserpine on riboflavin value fluctuations, amphetamine induces an initial large increase in riboflavin content which rises steeply to a maximum, over seven-fold of the control, at 8 hours. By 24 hours the value of riboflavin has reached control levels.

* Figs 1 a–g see Plate on page 98 b.

Comparing Figs 1, 2 and 3 we observe that in general the variations in melanin and riboflavin content of the liver at the different time intervals after reserpine and amphetamine follow a parallel course. However there is no absolute parallelism between the density of melanin in the section and the value of riboflavin in the tissue. For example, although the amount of melanin in Fig. 1 c (reserpine 8 hours) is similar to that of Fig. 1 f (amphetamine 8 hours) the value of riboflavin after amphetamine is three times higher than that after reserpine. We also notice that wherever the value of riboflavin is higher than the value of tetrahydrobiopterin, the liver is highly melanized (Figs 1 c, e, f).

Discussion

From our observations it is apparent that the administration of reserpine and amphetamine to *Triturus cristatus* adults affects the concentrations of both melanin and riboflavin in the liver. The changes observed after each drug indicate that the liver melanocytes respond to the drugs in a way similar to brain melanin neurons rather than skin melanocytes. Furthermore the drug producing Parkinsonism lowers initially the level of riboflavin in the liver while the drug, which alleviates the symptoms of the disease and counteracts reserpine sedation, *i. e.* amphetamine raises riboflavin to a value about 8 times higher than control.

In view of the fact that tetrahydrobiopterin is the cofactor for phenylalanine hydroxylation in the substantia nigra [13], our findings indicate that the levels of both riboflavin and tetrahydrobiopterin must be implicated in the demelanization and dysfunction of the substantia nigra neurons in Parkinsonism. The association of pteridines and riboflavin in the different organs of mammals has long been considered to represent a functional unit [14]. Since lumazines, which give rise to riboflavin through the action of xanthine oxidase [15] are formed in the rat liver as degradation products of tetrahydrobiopterin through the action of pterin deaminase, the value of riboflavin at any one time interval and its variations observed in our experiments will be the resultant of the relative activity of the above two enzymes. As pointed out by Rembold *et al.* [16] other factors as the O_2-dependence of the reactions is also affecting the balance of products. This O_2-dependence of the reactions may be the counterart of the histological findings that the normal substantia nigra neurons are in close contact with molecular oxygen and that the loss of this contact was the only detectable nigral lesion in patients with parkinsonian symptoms of recent date [17].

From our results we conclude that although tetrahydrobiopterin is the necessary cofactor for melanogenesis its variations have neither a direct or inverse correlation with the variations of melanin observed following the administration of the two drugs in this experiment. The same lack of correlation is observed also between melanin and isoxanthopterin. Thus it remains that riboflavin is the only one, among the three substances, which shows a correlation with the density of melanin in the liver of *Triturus cristatus* following the administration of reserpine and amphetamine. The type of involvement of riboflavin in melanogenesis at the moment is obscur.

Our results support the view that the mode of action of reserpine in production of parkinsonian symptoms and of amphetamine in the alleviation of such symptoms is through differential inhibition of the two (or more?) enzymes which connect the catabolism of tetrahydrobiopterin either towards isoxanthopterin or riboflavin.

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