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QUANTITATIVE CYTOCHEMICAL INVESTIGATION OF THE NINHYDRIN-SCHIFF REACTION

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Numerous methods are available for the histochemical detection of proteins. Some of these are applied for quantitative determinations. Among others, the Fast Green FCF procedure of Alfert and Geschwind (1953) and the Naphthol Yellow S-method of Deitch (1955) must be mentioned. Methods for quantitative histochemical protein determination on the basis of the arginine (McLeish et al., 1957; Sandritter and Krygier, 1959), tyrosine (Pollister and Ris, 1947), and SH-group content (Cafruny et al., 1955; Sandritter and Krygier, 1959) have been worked out by several authors. Attempts were also made at adapting the tetrazonium reaction to quantitative purposes (Benes and Sandritter, 1960). Some of these methods are suitable for the direct quantitative determination of basic proteins; some others may be regarded as general procedures for protein detection, being based on reactions of reactive groups of amino acids in the protein chain. If a number of alternative methods will be available for the same purpose, we shall have a better possibility to carry out quantitative investigations of the protein constituent of cells and cell components. In combination with the blocking of special groups and by the use of fractionated extractions, the quantitative determination of the various components of proteins will become possible.

In view of the possibilities discussed above a detailed quantitative study of the ninhydrin-Schiff and alloxan-Schiff reactions described by Yasuma and Ichikawa (1953) has been made. First, the specificity of the reaction was investigated. In further experiments those conditions and requirements were studied which must be fulfilled in quantitative cytochemical investigations. These are the absorption properties of the reaction product, the relation between layer thickness and colour intensity, the validity of Beer-Lambert's law, the relation of reaction time and colour formation after treatment with the Schiff reagent. On the basis of the results obtained, the ninhydrin-Schiff reaction seems to be suitable for quantitative cytochemical studies.

Materials and methods

The experiments were carried out with nucleated red blood cells and liver tissue, respectively. Adult *Amblystoma mexicanum* and *Pleurodeles waltlii* were decapitated and the blood collected was washed three times with saline without adding anticoagulants. Smears were then prepared and dried at room temperature.

The liver tissue of healthy adult white rats *(Rattus norvegicus)* was frozen in liquid air and fresh frozen sections were made from it in a cryostat. The thickness of the sections varied (cf. experimental part). Both smears and sections were treated with 10 per cent formaldehyde for 20 minutes. The preparations were then washed with running water for 20 minutes.

The ninhydrin applied was a 0.5 per cent solution in concentrated alcohol. The Schiff reagent was prepared according to de TOMASI (1936).

Quantitative estimation was made in a cytophotometer and a microdensitometer, respectively. The cytophotometer used (RAPPAY et al., 1961; RAPPAY, 1962) was suitable for point measurements within the limit of 1 micron. With a monochromator built in, the instrument could be used for the plotting of absorption curves. The electron-multiplyer attached (1 p 28 RCA) ensured the suitable sensitivity. The liver slices were subjected to direct densitometry by the use of a Joyce, Model MK III B microdensitometer.

Further details of the methods applied are described in the experimental part.

Experiments

In the first part of the experiments a quantitative analysis of the speci ficity of the reaction was carried out of the applying various blocking proce dures. The results of these experiments are summarized in Table I.

Table I

Inhibition of the ninhydrin-Schiff reaction of Axolotl blood cells

Blocking, Extraction	Composition	Temperature	Time	Result
Control	—	+20° C	16 hours	++++
Methylation	0.8 ml. cc HCl + 100 ml. methanol (Burstone, 1959)	+60° C	24 hours	++
Acetylation	15 ml. acetic anhydride 85 ml. pyridine (Burstone, 1959)	+37° C	4 hours	∅
Deamination	10 ml. acetic acid 5 g. $NaNO_2$ ad 100 ml. dest. water (Burstone, 1959)	+ 4° C	20 hours	∅
Extraction of DNA	5% trichloroacetic acid (Alfert and Geschwind, 1953)	+90° C	15 min.	++++
Blocking of aldehyde groups	10 g. Hydroxylamine HCl 20 g. Na-acetate 40 ml. dest. water (Burstone, 1959)	+20° C	2 hours	∅

It may be seen that no colour reaction was obtained after deamination and acetylation. Methylation of the carboxyl groups resulted in a decrease of the intensity of the reaction as compared to the controls. Similarly, no colour reaction appeared if the aldehyde groups liberated after oxidative deamination with ninhydrin had been blocked with hydroxylamine.

In addition to the blocking reactions, in further studies the deoxyribonucleic acids were extracted by treatment with 5 per cent trichloroacetic acid for 90 minutes (ALFERT and GESCHWIND, 1953). After extraction the colour intensity of the preparations was identical with that of the control.

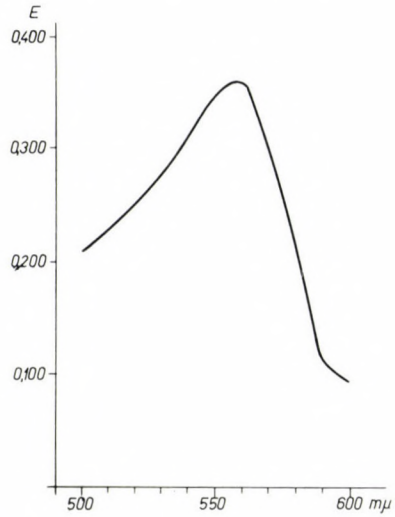

Fig. 1. Absorption curve of the reaction product

In the quantitative studies the absorption properties of the preparations were investigated. The absorption curve of Pleurodeles red blood cell nuclei measured cytophotometrically is shown in Fig. 1. The curve represents the

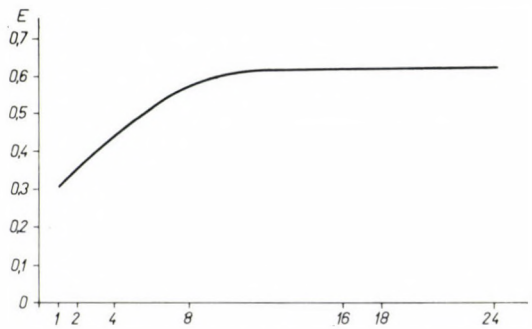

Fig. 2. Relation between duration of ninhydrin treatment and colour intensity in red blood cell nuclei

average of the absorption curves of five different nuclei. The absorption maximum is at 560 mμ. All the quantitative measurements were carried out at this wavelength.

After the establishement of the absorption curve the correlation between the duration of oxidative deamination with ninhydrin and the colour intensity was studied in smears and liver slices of equal thickness. The preparations were incubated for 0 to 24 hours with ninhydrin solution and samples were analysed cytophotometrically and densitometrically after 1, 2, 4, 8, 16, 18 and 24 hours.

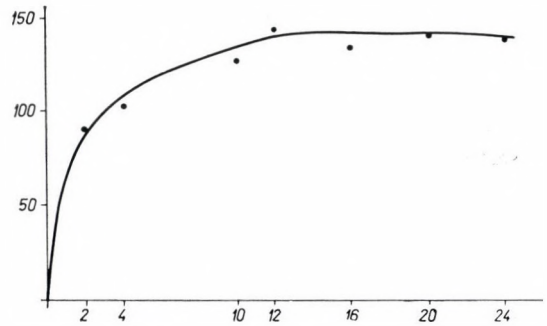

Fig. 3. Relation between duration of ninhydrin treatment and colour intensity in liver slices

The results of these experiments are shown in Fig. 2. (Abscissa: reaction time in hours; ordinate: extinction.) It may be seen that the colour intensity graddually increased and reached a maximum at 8 hours. Thereafter no change in colour intensity was observed.

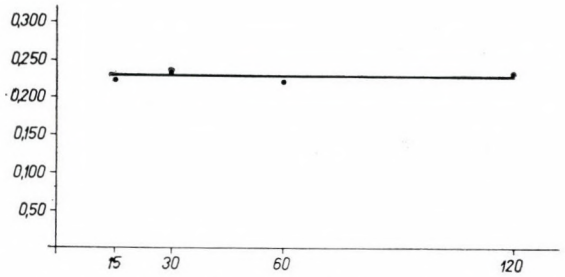

Fig. 4. Relation between duration of treatment with Schiff reagent and colour intensity in red blood cell nuclei

The behaviour of liver slices was investigated under identical conditions. These preparations were assayed densitometrically after treatment with ninhydrin for 2, 4, 10, 12, 16, 20 and 24 hours. The results are summarized in Fig. 3. (Abscissa: duration of treatment in hours; ordinate: colour intensity.) The trend of the curve is identical with that found with red blood cells.

It was supposed that in addition to the duration of ninhydrin treatment, the time of coupling with the Schiff reagent might also influence the colour

intensity of the preparations. Therefore, a number of Pleurodeles blood smears were treated with ninhydrin for 16 hours and the incubation with the Schiff reagent was varied from 0 to 120 minutes. The results are shown in Fig. 4. (Abscissa: time in minutes; ordinate: cytophotometric extinctions.) It is seen that the colour intensity remained the same from 15 to 120 minutes.

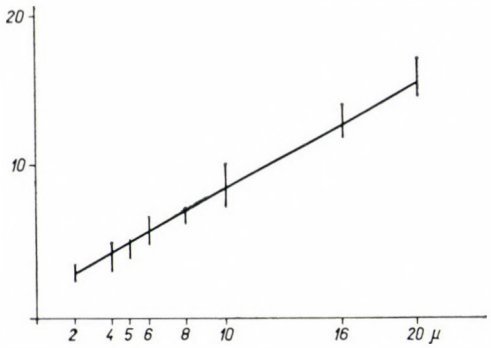

Fig. 5. Relation between layer thickness and colour intensity in liver slices

The relation between slice thickness and colour intensity was studied in liver slices. Sections 2, 4, 6, 8, 10, 16 and 20 μ thick were prepared in the cryostat, treated for 16 hours with ninhydrin and for 30 minutes with the Schiff reagent, and then subjected to densitometry. The results obtained are summarized in Fig. 5. (Abscissa: thickness of sections in μ; ordinate: colour intensity). It is seen that the colour intensity increased with the thickness of the sections.

Discussion

The mechanism of the ninhydrin-Schiff reaction is well-known. Due to the oxidative deamination of α-amino acids, relatively stable aldehyde groups are formed after treatment with ninhydrin (YASUMA and ICHIKAWA, 1953). The aldehyde groups reduce the leucofuchsin of the Schiff reagent and thus a red colour results. It has been supposed that the α-carboxyl groups also participate in the reaction (BURSTONE, 1955, 1959), an assumption supported by the present studies as well. It has namely been found that the intensity of the reaction is decreased after the blocking (methylation) of carboxyl groups. The specificity of the reaction has been proven by the blocking procedures applied in the present studies, and by the extraction of deoxyribonucleic acids. Blocking of the corresponding amino groups or that of the liberated aldehyde groups equally inhibits the reaction.

On the basis of the specificity and technical simplicity of the reaction, it seemed worthwhile to study its quantitative properties. The application of a protein reaction in which the colour formation is evoked by the Schiff reagent offered distinct advantages, as the amount of deoxyribonucleic acid (Feulgen reaction) and proteins (ninhydrin-Schiff reaction) can be estimated under identical conditions. First of all, the identity of absorption maxima given by the ninhydrin-Schiff and Feulgen reactions should be stressed.

Reactions used in quantitative cytochemistry, in addition to their specificity, must under given stoichiometric conditions follow the BEER-LAMBERT law, which means that the extinction measured must be proportional to the increase in layer thickness. This direct proportionality has been clearly established in our studies on liver slices. In addition, it had to be taken into account that the reaction is not immediately complete, the deamination with ninhydrin and the coupling of aldehyde groups with fuchsin takes a certain time. The results presented indicate that the optimal duration of treatment amounts to 14 hours and that of the staining to 15 minutes. Subsequently the colour intensity did not change, at least not in the material used in our experiments.

The quantitative measurements were repeated with the same experimental material several times. The comparison of the results of consecutive experiments indicated a good reproducibility.

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Summary

The ninhydrin-Schiff reaction has been subjected to quantitative cytochemical studies in amphibian red blood cells and mammalian liver tissue. The reaction has been found to be specific, well reproducible and to follow the Beer-Lambert law. It is thus thought suitable for the quantitative study of α-amino acids.

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QUANTITATIVE ZYTOCHEMISCHE UNTERSUCHUNG DER NINHYDRIN-SCHIFF'SCHEN REAKTION

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Es wurden quantitative Untersuchungen der Ninhydrin-Schiff'schen Reaktion am Blutgewebe von Amphibien und am Lebergewebe von Säugetieren durchgeführt. Es wurde festgestellt, daß die Reaktion spezifisch und reproduzierbar ist, dem Beer-Lambert'schen Gesetz folgt und somit für die quantitative Untersuchung der α-Aminosäuren geeignet ist.

КОЛИЧЕСТВЕННОЕ ЦИТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ РЕАКЦИИ НИНГИДРИН-ШИФФА

ДЬ. РАППАИ и Й. ТЁРК

Проводилось цитохимическое исследование реакции нингидрин-Шиффа в крови земноводных и в печеночной ткани млекопитающих. Было установлено, что реакция является специфической, репродуцируемой, следует закону Беер—Ламберта и, следовательно, пригодна для количественного исследования α-аминокислот.

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