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OBSERVATIONS ON SOME CYTOCHEMICAL REACTIONS FOR NUCLEOPROTEINS

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Résumé—Deux méthodes histochimiques sont décrites pour l'étude d'une fraction protéinique que est liée à l'acide déoxypentose-nucléique. Il est démontré comment une de ces méthodes permet des mesures quantitatives de la teneur en cette protéine par microphotométrie.

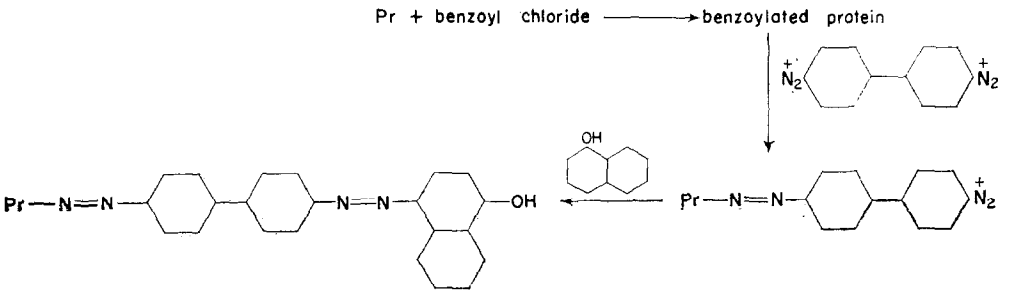
IN THE field of protein cytochemistry there is a tremendous number of potential approaches, most of which have not been worked out properly, so that one knows surprisingly little about the cytochemistry of proteins compared with the cytochemistry of deoxyribonucleic acid (DNA). One of the basic reasons for this is that although it is quite simple to think up potential good reagents for protein cytochemistry, the amount of analytical work which is necessary before these can be used effectively is forbidding.

The principles which I think are essential with many techniques, and which we have been developing in my laboratory, are as follows: first one chooses a chromogenic reagent. This should preferably be a highly-diffusible compound. Secondly, one chooses one or more blocking agents. The reason why this is necessary is that, practically speaking, there are no chromogenic chemical agents which are of sufficient specificity to enable one to detect just one grouping in a protein. But an increase in specificity can be obtained by first blocking up some of the groups with which a chromogenic reagent may react. The third essential is analytical work, with two purposes in mind. The first is to find out what groups have reacted, because although one can predict on general chemical grounds what any particular compound such as the diazonium hydroxides or fluorodinitrobenzene ought to do, in fact what will actually happen when such a reagent is applied to a tissue section or a smear is uncertain, and therefore one has to carry out analytical work to find out what has actually reacted as opposed to what one hopes has happened. Secondly, if the technique is to be used for quantitative work, one has to find out whether the reagent has reacted quantitatively.

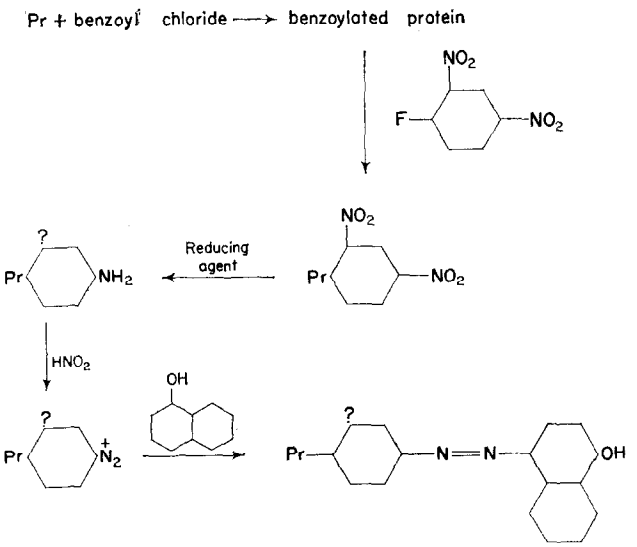
As examples of chromogenic reagents, I shall take diazonium hydroxides and fluorodinitrobenzene (FDNB). As an example of blocking agents, benzoyl chloride, perhaps one of the simplest, we find useful. The easy part of this type of investigation is to think up alternative compounds which will act as chromogenic or blocking agents, whereas the solid work needs to be put in on the analytical side. It is an interesting thing, on looking through the literature, to find that for every paper on the analytical side there are about a hundred on new reagents or untested reagents! That is exactly the opposite of the desirable distribution.

Strictly speaking, benzoyl chloride ought to block or to destroy all those components of protein which will react with diazonium hydroxides or with fluorodinitrobenzene. In fact this does not happen. Not all of the potentially-reactive groups are blocked. We have investigated the groups in tissue sections which are protected in some way against the action of benzoyl chloride. The sequences of chemical reactions which we have therefore used are the following, in which Pr indicates protein in a tissue section.

Benzoyl-diazo procedure



Benzoyl-FDNB procedure



Examination of slides treated by either of these techniques shows that, in ordinary frozen-dried tissue sections, practically all groupings in the cytoplasm, potentially capable of reacting with diazonium hydroxides or with FDNB, are blocked or destroyed by benzoylation. But in the nuclei, although most of the potentially-reactive groups are eliminated, some remain untouched by benzoyl chloride but accessible to diazonium hydroxides or FDNB. The distribution of colour in nuclei is similar to that of DNA, and with *Drosophila* salivary chromosomes the reactive components are restricted to the Feulgen-positive bands.

One can conclude from these preliminary observations that after benzoylation there are some groups in proteins, competent to react with diazonium hydroxides and competent to react with fluorodinitrobenzene, which are found only in the nuclei and in one or two cytoplasmic sites; and in particular, in the nuclei the distribution of these components is similar to the distribution of DNA. This led us to believe, or perhaps I should say suspect, that we were dealing with a reaction which was specific for some type of nucleoprotein. We therefore set to work to analyse chicken erythrocyte nuclei. The work on the diazo reaction was done by Dr. E. BARNARD and on fluorodinitrobenzene by Dr. A. MADDY. The principles of this study were to take nuclei, and remove the lipid from them. When nuclei have been treated with diazonium hydroxide there is no colour in the lipid. With FDNB there is a little residual colour, that is to say some reaction has taken place between lipid and FDNB even after benzoylation. We have not followed that up, though we may do later. This leaves one with lipid-free nuclei, and on fractionation of the lipid-free nuclei one finds that the colour is associated with the DNA-protein fraction. There are other protein fractions which can be removed from the nuclei which have no colour associated with them. Finally, when the protein and the DNA of the DNA-protein are separated, the colour is found exclusively with the protein. So one ends up, in the case of material which has been treated with diazonium hydroxides, with a protein which contains somewhere in it some amino acids which are part of an azo dye.

The next step was to hydrolyse this protein and separate this component linked to azo dye from all the other amino acids. On hydrolysis the usual collection of amino acids are obtained, plus this component. Now the behaviour of the azo component tends to be dominated by the azo dye end of the molecule, and this makes it relatively easy to separate this from all the other amino acids. Then one can identify this component. First the azo group is reduced. When this is done carefully, three low molecular weight components are obtained, of which in the simplest case the component to be identified will be the amino derivative of the initial amino acid. It should therefore be relatively easy to identify by various standard techniques. In the case in question, it was not easy to identify because the component we were interested in was unstable and decomposed readily. However, we were eventually able to chase it down and proved by synthesis, by study of its behaviour on paper chromatography and by infrared examination, that the primary product that is released on reduction is amino-histidine. It was thereby shown that when the benzoyl-diazo reaction is carried out with benzoylated sections, the only component in the protein which does in fact react with the diazonium hydroxide is a fraction of the histidine. Not all the histidine in the nuclear protein fraction is protected against diazonium hydroxide: only a small fraction of the total histidine is so protected.

A similar series of operations was gone through with fluorodinitrobenzene and in that case what turned up in the fraction which was finally available for analysis was tyrosine, histidine and some traces of other amino acids. This means that in the nucleus of the cell there is protein in some sort of relationship with DNA such that some of the tyrosine and some of the histidine are protected against the action of a relatively violent reagent such as benzoyl chloride (and as a matter of fact,

against other blocking agents too). There are small amounts of other amino acids which have turned up in the fluorodinitrobenzene treatment, and we cannot yet evaluate the significance of this.

The next question which arose was whether it was possible to make any quantitative estimation of the amount of these reactive components per nucleus. Dr. E. BARNARD has worked this out recently in the case of the diazo compounds. He found the best material was frozen-dried sections or smears and he measured the absorption with DEELEY'S apparatus. With mouse sperm heads there is a sharp single mode, rather as one would expect, around twelve to thirteen arbitrary units of the stain per nucleus. With mouse liver there are the usual three classes of cells which correspond actually to the diploid, tetraploid and octaploid hepatic cells. In addition there is a mode due to another group of cells which are not hepatic cells. The amount of stain in arbitrary units in the diploid cells is far more than twice that found in the sperm; that is to say, far more than twice the haploid amount which was found in the sperm. With mouse kidney a single mode is obtained for proximal tubule cells which is actually about ten times that found in the sperm, compared with twice which one would expect if the relationship between this protein fraction and the amount of DNA were constant. Further, if one compared one animal with another, so far as we have gone, with sperm the amount is constant within a species. Also, with nucleated red blood cells, the amount is constant within a species, and with cells of the same degree of ploidy in the liver, the amount is constant within a species. But with the kidney, which is the other tissue we studied, there is very great variation from one animal to another within a species. In other words, this protein fraction seems to be extremely variable in the kidney. There may be other organs in which it is extremely variable too.

We have no chemical information as to what sort of protein is involved in these reactions, other than that it contains histidine and tyrosine. The next really important thing we have to investigate is the turnover number of this protein, because it remains to be seen whether it is a structural component of the chromosomes. If it is not a structural component, then I think it is fairly obvious that it must be one of the first gene products. If so, it probably has a very high turnover number and we should be able to decide between these possibilities simply by measuring the turnover number.

SUMMARY

Two histochemical methods are described for studying a protein fraction which is bound to deoxypentose nucleic acid. One of these methods has been shown to permit quantitative estimation by microphotometry of the amount of this protein.

DISCUSSION

P. ALEXANDER: Many instances are known where groups are sterically inaccessible in native proteins and the proportion that are inaccessible varies with the reagent used. This differential availability gives you the staining you see. On denaturation these groups become accessible but the severity of the denaturation treatment needed to reveal these groups varies widely from protein to protein. Would it be that the protein which you stain is particularly resistant to denaturation —possibly the presence of the DNA serves to stabilize it against the denaturing action of the fixation procedure?

J. DANIELLI: We are not sure to what extent the "unmasking" of histidine in our experiments is analogous to "unmasking" arising during protein denaturation. This is obviously one possibility. But another is that a lipid is involved.

J. BRACHET: Have you tried to analyse your protein by fingerprinting after tryptic digestion in order to find out whether the histidine and tyrosine which have reacted are localized in certain specific peptide sequences?

J. DANIELLI: No, we have not. But it will be well worth doing.

F. KASTEN:

(1) It is very gratifying to learn that Dr. DANIELLI has been doing cytophotometric work. However, I wonder if the application of this technique to this protein-staining reaction does not require some basic work. For example, tests for adherence to the Beer–Lambert law, constancy of absorption-curve shape in different nuclei, etc.

(2) Although your measurements of protein in certain tissues corresponds with DNA content, it seems anomalous that the kidney protein values differ so markedly, since this is a diploid DNA population.

(3) Have you tried removing DNA and noting the effect of the protein?

J. DANIELLI:

(1) As pointed out in my paper, it is Dr. E. A. BARNARD who is responsible for the cytophotometry. Some of the basic investigations have already been made by Dr. BARNARD. Others will have to wait until we have identified the protein involved.

(2) Until we know what function is performed in the cell by the nucleoprotein we are studying, it is difficult to say what is, and what is not, anomalous. The variation observed in diploid kidney cells does, as you say, indicate a fruitful point for further study.

(3) No.