Histochemical aspects of nucleoproteidic metabolism of human embryos

A histofluoroscopic study

M. PELLICANO

Summary: The Author, after stressing the importance of proteic syntheses in the vital processes of each cell and the role played by nucleic acids in such metabolic processes, underlines the importance of histochemical studies on nucleoproteidic metabolic in the embryo, and briefly illustrates the most important features of the fluorescent method.

The Author further relates the results of his personal research conducted by means of microfluoroscopy after acridine orange chromization on histological sections of human embryos.

The results are documented by the enclosed photographs, and their significance is briefly discussed.

Key words: Nucleoproteidic metabolism; Human embryos; Histofluoroscopy.

INTRODUCTION

The study of nucleic acids in relation to their role in carrying out cellular metabolism and their importance in the phenomena of multiplication and differentiation of the cell through protein synthesis, has been given a lot of attention and has led numerous experts to devote themselves to this subject.

The evaluation of these processes, which have proved to be of constantly increasing importance in every tissue and in every normal and pathological condition, has been carried out through biochemical and histochemical studies: Feulgen reaction, toluidine blue, methyl green and pyronine methods, overvital coloration with neutral red, thyonine method, and ultraviolet photometric and spectrophotometric dosages, based on the observation that nucleic acids show a maximum of absorption to the ultraviolet at 2560 Å, and, last, with the use of tracer isotopes.

Fluorescence is the property possessed by certain substances to release, through short wave-length electromagnetic radiation, a visible radiation, usually of a longer wave-length; nevertheless, the colors of the light radiation released by primary fluorescence are often so dim and of such weak intensity as to prevent an analysis and a clear definition of the fine tissue structures. Therefore, to achieve this goal the tissues were coloured with highly

Received 10-5-1994 from the
Institute of Human Anatomy
Medical School
University of Naples "Federico II", Italy

Revised manuscript accepted for publication 10-9-1994.

fluorescent substances tying them to the tissue molecules without changing their structure ([1]).

Fluorochromes are organic composites that react chemically with the substrate without alterations and give a clearly observable fluorescence in the elective areas of endocellular location. They are used in very diluted solutions (1 : 500 or 1 : 10.000); thus, without damaging the solution, they are preferred to the common vital colourings, effective only at relatively high concentrations.

The biological substances' affinity to fluorochromes is highly specific, and because of this the various fluorochromes are used when related to the subject of study.

Several possibilities of histochemical use of composites with secondary fluorescence are given by the process of fluorochromization to identify nucleic acids (type ribo and desossiribo), mucopolysaccharides and lipids as such and in lipoproteidic complexes ([1, 2]).

Basic fluorochromes color the cellular structures including the nucleus and, once tied to the various cells' acid radicals, give out fluorescence of different colour by metachromasy ([2, 3]).

Metachromasy is an often occurring phenomenon in fluorochromization: different areas of the same preparation get different colouring through the same fluorochrome, with regard to quality and quantity as related to their chemical structure. This characteristic allows for a topochemical study on particular substances in cell-tissue areas.

A particularly important fluoromicroscopical technique among those applied to the histochemistry of proteins and lipids is fluorochromization with acridine orange, a basic colouring substance of the acridine series (2.8-dimetilamino-acridine), stable from pH 1.2 to 6.6, which in solution releases a green leaf-type light, while if absorbed gives out a red copper-like fluorescence. Its cation has a characteristic metachromasy of concentration, since it offers a green fluorescence at low concentrations and a red fluorescence at high concentrations, this being a phenomenon that, empirically named "concentration effect", is due to the formation of polymer cations with a red fluorescence: the different levels, of substrate polymerization (nucleic acids) in tissues which the colouring substance combines with, induces the different degrees of polymerization of the colouring itself, and, therefore, the metachromasy.

Based on the most recent findings, it can be said that, using weak concentrations of acridine orange (0.01%), the nuclei, given their desossiribonucleic acid (DNA) content, show a green fluorescence (might go into pale yellow), while the cytoplasm, due to the RNA content takes on a red metachromatic fluorescence (might to into brown, reddish-brownr or orange). The intermediate colors of yellow and orange, especially noticed in the nucleolus and perinucleolus areas, might be due to variable concentrations of the green fluorescent monomere and the red fluorescent polymer cations.

The verification by researchers of a relation between tissue growth and metachromatic intensity has lead to the belief that chromotrope substances take on a biological function concerning the growth and regeneration of the tissues themselves. Their presence is plentiful in the embryonal connective tissue and particularly in rapidly developing tissues: primitive eye, eyelid, tooth outline, piliferous follicle, etc., they decrease with the differentiation of the tissues, disappear at the end of the growth, and reappear in regeneration processes after undifferentiation: regeneration of the epidermis, the uterine mucosa, the nerves, etc. ([4]).

The very high versatility degree of the structure and the ubiquitary spread of the nucleic acids make up the basis of their

essential importance in biological phenomena: the desossiribonucleoproteins are indeed the basic gene constituents and are, therefore, closely tied to the phenomena of cell reproduction; in fact, in the dividing cells the nuclear system has an intense activity, and a great quantity of highly polymerized DNA is present.

In the proteic biosynthesis process the interested ribonucleic acids are: messenger RNA (mRNA), which has the information for the characteristic sequence of aminoacids within the proteic molecule; soluble RNA or transfer (sRNA), which brings activated aminoacids onto the ribosomes (enzyme-aminoacil-adelinate complex), and ribosomial RNA (rRNA).

In short, the DNA and RNA functions and those of their proteic complexes, can be summarized like this: a stable DNA, at a very high degree of polymerization and with a slow metabolism, is mainly given the role of conservation and transmission of the genetic supply; under its direction RNA acts, a dynamic entity, with a fast metabolism, easily influenced by environmental conditions and functional requests, particularly delegated to single cell protoplasm reconstruction, that, as a "messenger", "programmes" ribosomes for synthesis of particular proteins.

From a histo-fluoroscopic point of view, in cells poor in RNA (superficial layers of the flat epithelium) the cytoplasm will either be green or not have fluorescence; in cells with a light RNA content (cells of the intermediate layers of the flat epithelium, macrofages) the cytoplasm will have a brown color; cells with an average RNA content (basal cells, epithelia of the respiratory tract, cells of the cervical channel) have a reddish brown color ([4, 5]).

Thus, the RNA content of different organs presents a considerably wide range: some organs, very active physiologically such as the heart, the voluntary muscles, and the kidney, have a small quantity of it; on the other hand, organs and tissues, whose cells enjoy intense reproduction, are exceptionally rich in ribonucleic acid: blood matrix cells, Lieberkühn crypt cells, generating hair and the epidermis, etc. ([6, 7]).

Those rich in nucleic acids are:

1) Intermitotic vegetative cells, undifferentiated. They multiply continuously (epidermis basal cells, haemocytoblastes, spermatogones), producing identical cells or cells that will later undergo differentiation. Their life span is the interval between two karyokinesis.

2) Intermitotic differentiated cells that, between one karyokinetic process and the next, undergo a proressive differentiation (differentiation of epidermic cells, spermatogonium in a spermatozoid, haemacytoblast in a mature haematic cell).

As in the previous case, their life span is the interval between two karyokinesis.

The quantity of nucleic acids varies in the cells during embryonal development and the cell's vital cycle. Applying the Feulgen method during segmentation, it is possible to observe the constant enrichment of DNA nuclei; in this way, in the cells vital cycle, we have a maximum DNA synthesis with the formation of chromosomes in the promethafase.

The growing ovocytes contain plenty of DNA at the time the proteic synthesis of the vitelline body begins. During embryonal development, there is a synthesis of DNA the moment each organ is formed; then, it decreases when histogenesis begins. It is known that in a chicken egg an intense synthesis of the desossi and ribonucleic acids takesplace during the egg's development.

The situation is not quite as clear for those eggs with less yolk, like those of frogs. The rate of ribonucleic acid of frog eggs stays constant during segmentation and the beginning of gastrulation; a synthesis of RNA begins to take place during the last one, and becomes fast and plentiful when the nervous system develops.

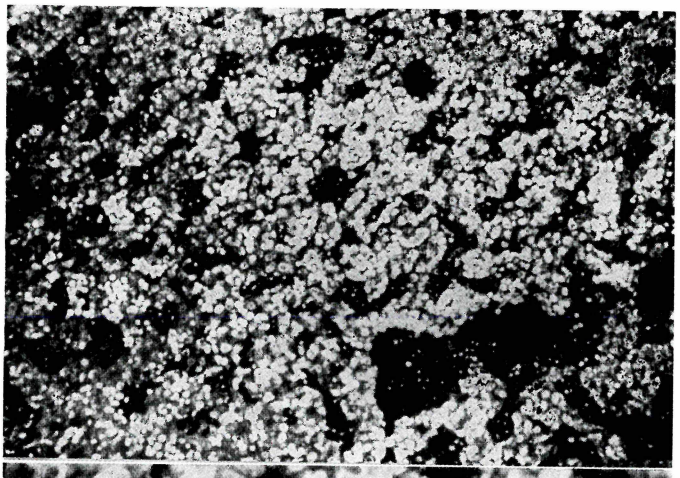

Fig. 1. — Liver (100×).

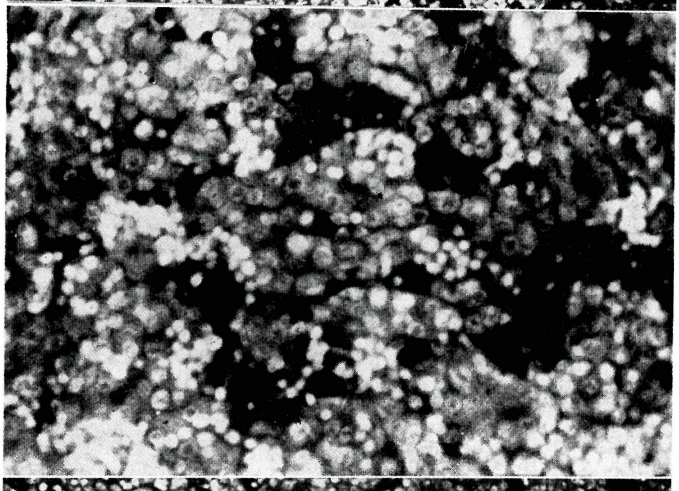

Fig. 2. — Liver (250×).

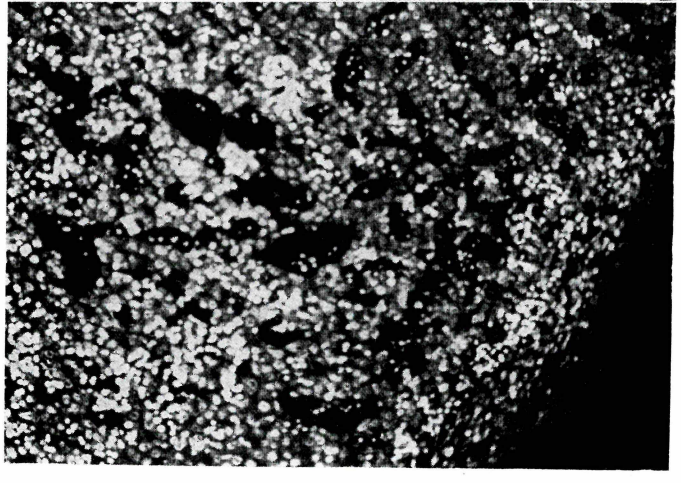

Fig. 3. — Liver (100×).

During gastrulation the RNA load of the vagina material decreases, while it increases progressively in the cells that make up the neural plate.

At more advanced levels, each organ becomes rich in ribonucleic acid the moment when its differentiation begins: basofily decreases when cytological characters become evident, that is, when the characteristic proteins of every tissue have been greatly synthesized. Yet, this secondary decrease of basofily is lacking in cells that will keep on synthesizing large quantitiesof proteins later on, like the liver and pancreas.

MATERIALS AND METHODS

We have had the chance to carry out a microfluoroscopic study on two human embryos, one of which was 14 mm in length and had been taken immediately after periscopy for ectopic pregnancy, and the other was 16 mm long and had been found in an abdominal cavity due to rupture of the pregnancy tube.

This study offers a detailed description of fluorescent structures of tissues and provides an interesting fact on histochemical morphology of embryonal tissues. Furthermore, the intention was to carry out a thorough examination on the relation between fluorescence and some cell functions, since the specificness of the microfluoroscopic method after chromization with acridine orange shows in an elegant way the possible quali-quantitative variation of the nucleoproteidic cell situation.

The embryos, one of which was covered with its membranes, were immediately all fixed in ethyl alcohol at 70° at 4° C for 24 hours (researchs in microscopy of fluorescence, especially those conducted with histochemical goals, assume that the tissue be fixed before post-mortem modifications can occur).

A longitudinal median cut, was then done on each of the two embryos and the two halves, after dehydration and diaphanization in xylol, were kept in xylol-paraffin in a thermostat at 37° for 12 hours; later in paraffin at 42° for 4 hours and then enclosed in titrated paraffin at 56°.

Strict compliance with this schedule is very important because if one were to use paraffin at a high melting point, phenomena of DNA depolymerazation and disappearance of RNA would be induced, such as those obtained after treatment with acids.

The very fine sections, after being deparaffined with xylol, were put in distilled water and then subjected to fluorochromization. Acridine orange was used at a concentration of 0.01% in a tampon of phosphates 1/15 M and pH 6. It was necessary to use unfluorescent slides, since even the slightest presence of impurities determines the sticking of a very fine film of colouring giving the field a fluorescence of an unspecific pink background, which disturbs the observation.

RESULTS

Reported are the obvious and more characteristic traits of organs and embryonal tissues after histofluoroscopic observation.

Liver. Parenchyma cells show a bright orange-red fluorescence for the cytoplasms and nuclear yellowish green tone Vasal endothelia close to the hepatocites show a fine intense-red metachromatic leaf (Fig. 1 - 2). Fairly evident is the bright green fluorescence of the nuclei of the pavement-wise mesothelial cells (Fig. 3).

Lung. A crimson-red cytoplasmatic fluorescence stands out in the bronchial and bronchiolar epithelium, together with a nuclear golden fluorochromic tone due to the interference of the red cytoplasmatic fluorescence with the green one of the nuclei. Vasal formations can be seen which are formed by a thin fibrilous metachromatic ring intervally covered by a brigh red fluorescent endothelium (Fig. 4).

Heart. An orange-red intense fluorescence stands out at the cytoplasm level and a yellow nuclear one of interference of the muscular cells. The endothelium covering the ventricular and atrial wall shows a slightly more marked fluorescence (Fig. 5).

Pancreatic outline. The cell cords derived from the endoblastric epithelium branch out in the mesenchyma, gaining a tubular shape. A bright red cytoplasmatic fluorescence and a golden nuclear of interference stands out at the epithelium leval. Some vasal formation can be seen

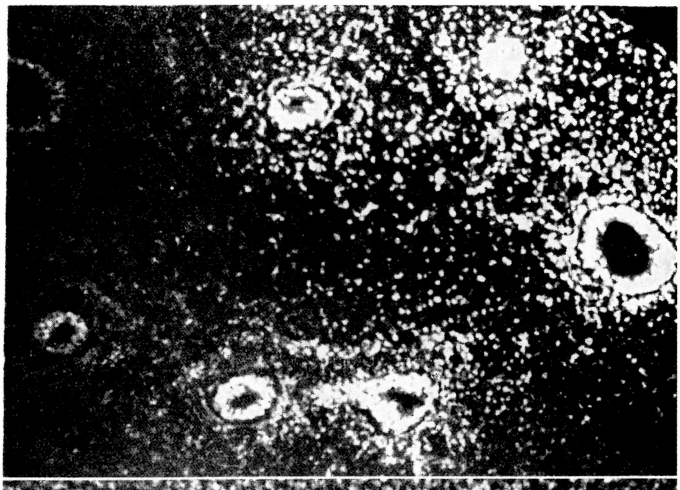

Fig. 4. — Lung (100×).

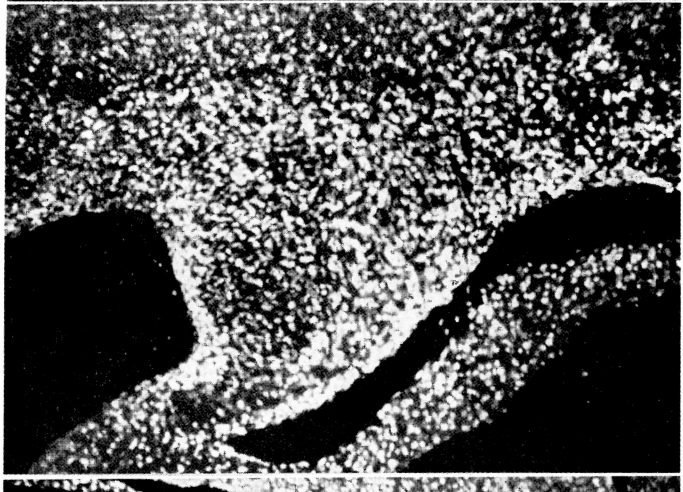

Fig. 5. — Heart, trabecular and cortical part ((100×).

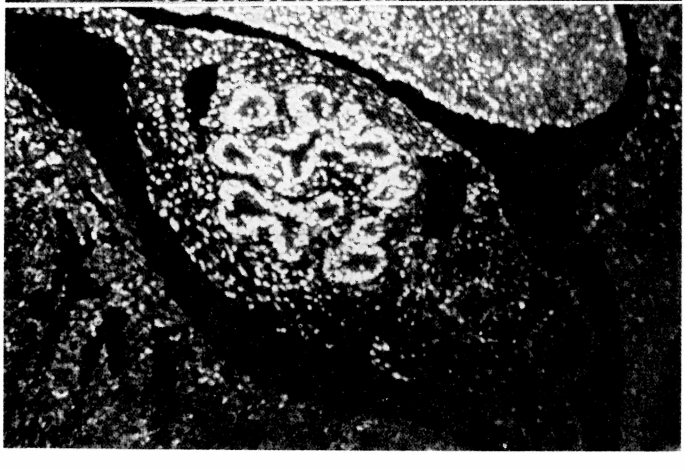

Fig. 6. — Pancreatic outline (100×).

consisting of a thin yellow-green fibrilous metachromatic ring internally covered by endothelium and linked to the nearest stroma (Fig. 6).

Stomach. A flame-red cytoplasmatic fluorescence and a golden nuclear tone of interference stand out at the epithelium level. In the musculature vasal formations can be seen, made up of a thin metachromatic fibrilous ring internally covered by a red fluorescent endothelium and linked with the surrounding stroma (Fig. 7).

Intestine. An intense cytoplasmatic flame-red fluorescence can be seen at the mucosa level, indicating a high RNA content, contrary to the nuclear golden tone of interference (Fig. 8, 9, 10).

Peritoneous mesothelium. A bright red fluorescence stands out at the cytoplasm level and a yellow-greenish fluorescence at the nuclei level (Fig. 11).

Trachea. A brick-red cytoplasmatic and yellow-greenish nuclear fluorescence can be seen at the mucosa level. A clear nuclear green fluorescence stands out at the wall structure level (Fig. 12).

Neuraxis. The cytoplasm of the ependimal cells shows an orange-red fluorescence, the same as the cytoplasm of the marginal layer cells. Neuroblasts and spongiblasts piled up together have an intense nuclear golden fluorochromia of interference. On the outside in fluorochromized preparations the coating of the the tegumental ectodermis can be seen where a nuclear yellowish-green fluorescence stands out (Fig. 13).

Marrow body. The ependimal epithelium shows a bright-red cytoplasmatic fluorescence which tends to fade towards the marginal layer. The neuroblast nuclei take on an intense yellowish-green fluorescence (Fig. 14).

Spinal column. The different tones of fluorescence stand out at the level of outline of vertebral bodies and of the intervertebral discus. In the middle the spinal cord appears covered by its sheath. The cytoplasm shows a red fluorescence and the nuclei a yellow one (Fig. 15).

Coriodeous plexuses. The cell elements show a crimson-red fluorescence at the cytoplasmatic level and a golden hue of interference at the nuclear level (Fig. 16).

Costal processes. A crimson-red fluorescence at the cartilaginous cell level and a fluorochromic nuclear golden tone of interference is evident (Fig. 17, 18, 19).

DISCUSSION AND CONCLUSIONS

The characteristics recorded through histofluoroscopic observation after chromization with acridine orange at the embryonal level, lead to some interesting considerations.

One consideration when weighing the physical parameter of fluorescence in histochemistry, concerns the possibility of fluoromicroscopy from a qualitative and quantitative point of view. A first exam can already give us some indication on the quantity of a given substance or a mix of fluorescent substances in a given cell/tissue structure, morphologically well-defined, through a relative dosage of the intensity. Keeping experimental conditions constant (temperature, nature and intensity of the exciting radiation, of the middle pH, etc.) there exists, within limits, direct proportionality between intensity of fluorescence and quantity of substance present.

Opportunely using acridine orange as fluorochrome, the disossiribonucleic acid of the nucleus turns green or greenish yellow, while the ribonucleic acid of the cytoplasm, at increasing concentrations, takes on a range of colouring from brown, reddish-brown, to orange and flamered.

This phenomenon is more evident as the nucleic acid content increases: in fact the characteristics of the tissue structures which the caution of the acridine orange

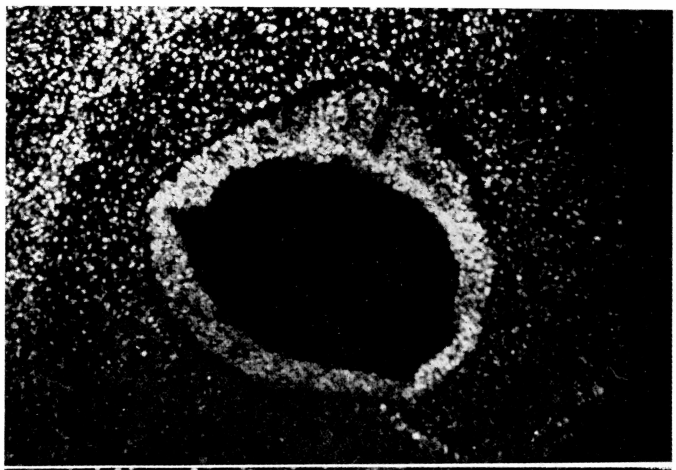

Fig. 7. — Stomach (100×).

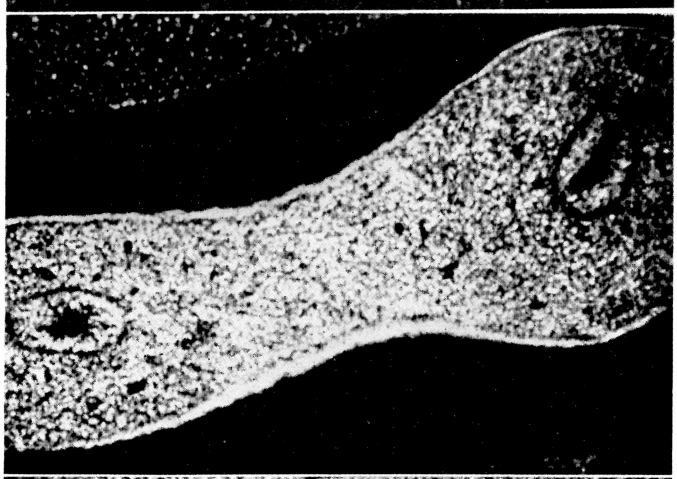

Fig. 8. — Intestine (100×).

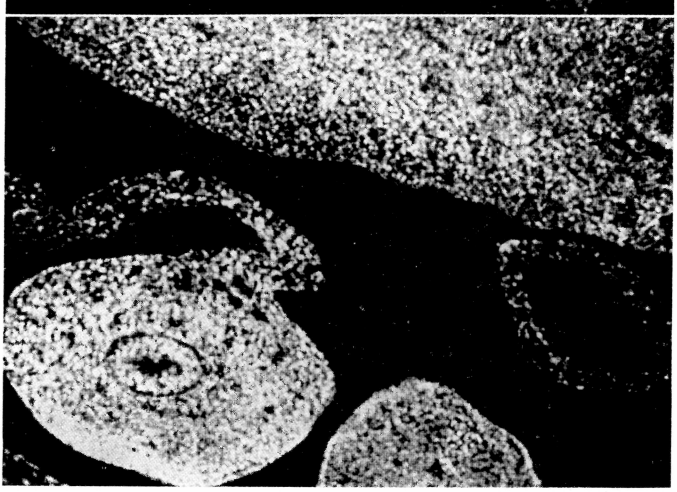

Fig. 9. — Lengthwise median section regarding, on the left, an intestinal loop with its mesenteriolis and, on the right, the onfalo-enteric vein (100×).

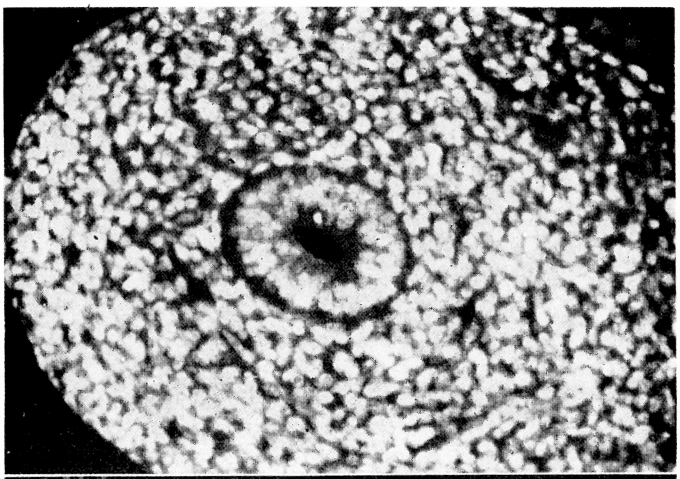

Fig. 10. — Intestine (250×).

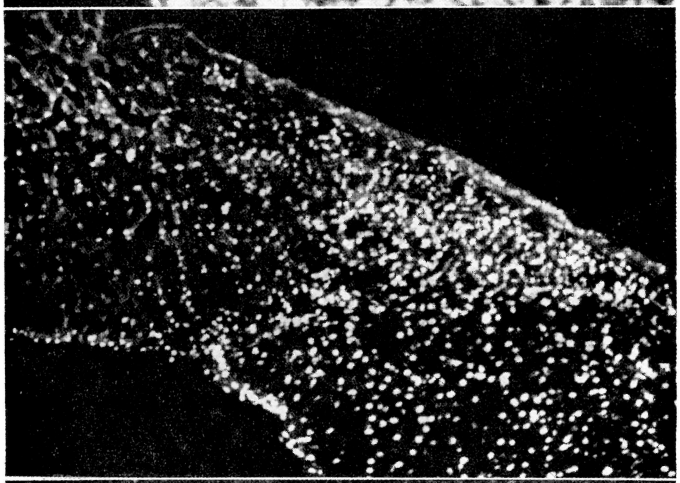

Fig. 11. — Peritoneal Mesothelium (250×).

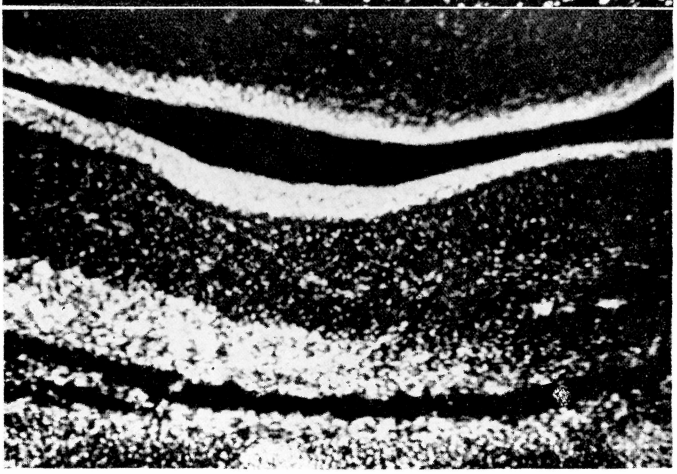

Fig. 12. — Lengthwise section at the esophagus and trachea level (100×).

Fig. 13. — Neuraxis (100×).

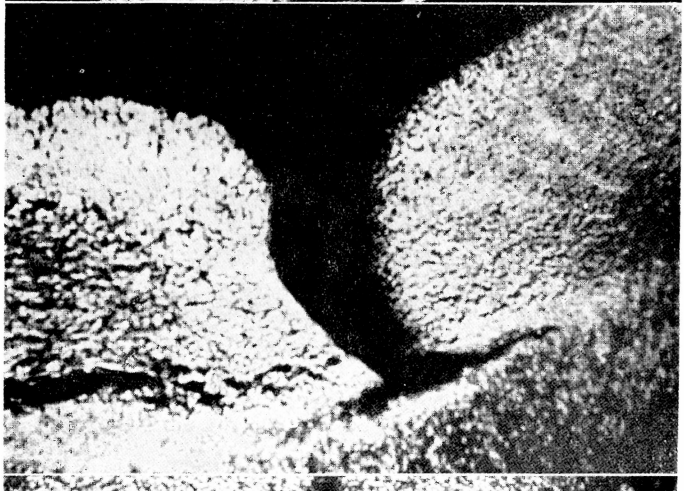

Fig. 14. — Marrow body (250×).

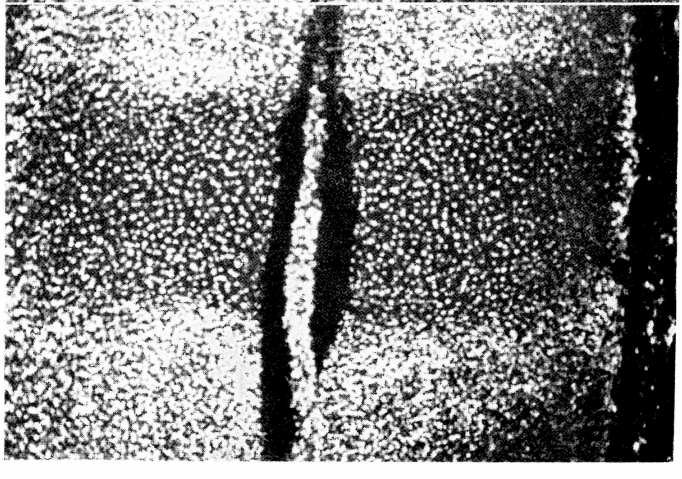

Fig. 15. — Sagittal median section at the spinal column level (100×).

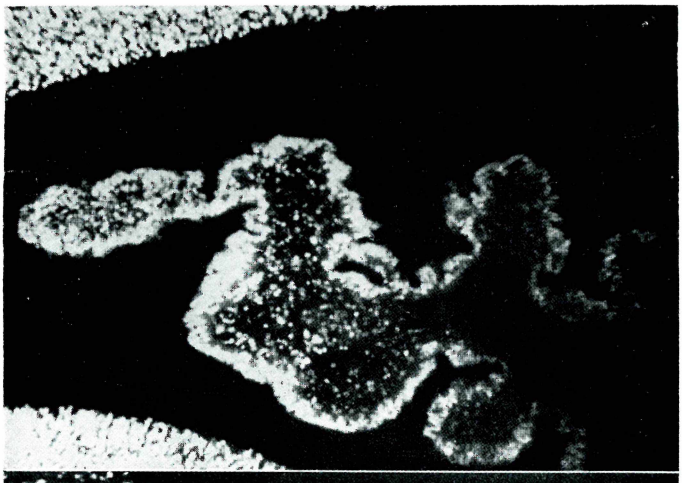

Fig. 16. — Choroid plexuses (100×).

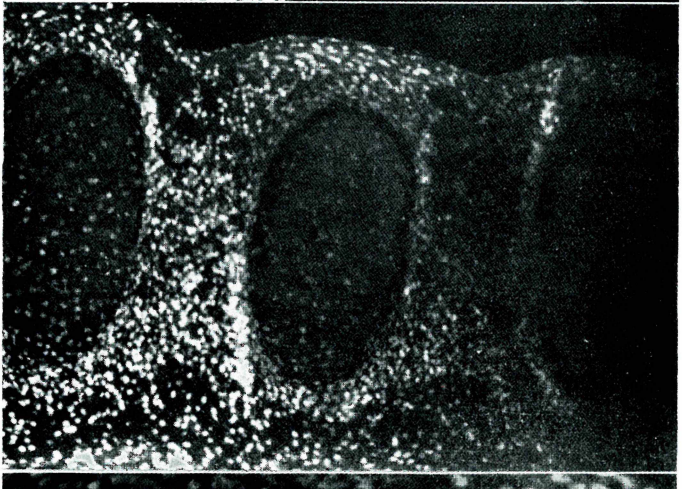

Fig. 17. — Sagittal section regarding three costal processes (100×).

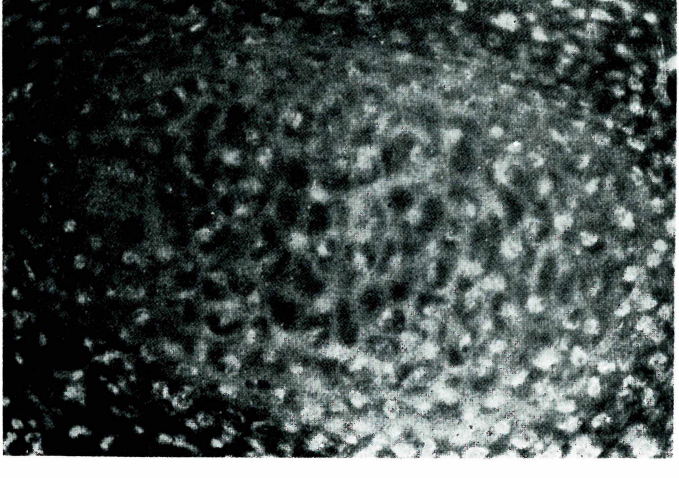

Fig. 18. — Costal process (400×).

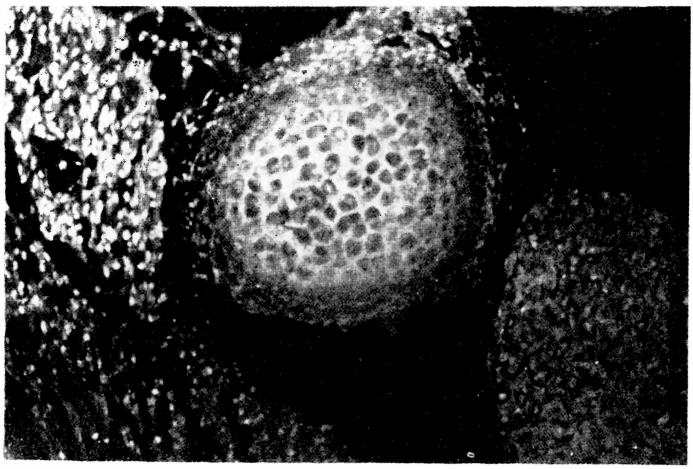

Fig. 19. — Lengthwise section concerning in the middle a costal process, on the left a trabecular and cortical part of the heart and, below on the right a part of the liver area (100×).

is fixed on, cause more or less absorption of fluorochrome by the tissues, thus determining the different reactions with respect to the issuing of light.

It is necessary to underline that in all cases examined, the treatment of the sections with RN'ases has eliminated every red cytoplasmatic colouring, establishing, besides the presence and the interaction of the RNA with the fluorochrome, also the absence of acid polysaccarides capable of falsifying the fluorochromatic aspects.

The study based on fluoroscopy after chromization with acridine orange shows consistently the importance nucleic acid has in the reproduction and the growth of cells; in fact, it clarifies the various biological and functional behaviors of the tissues examined.

In the process of proteic biosynthesis, DNA acts as an inductor in the formation of RNA; this, in turn, shapes the proteosynthesis at a speed proportional to its concentration. Since DNA is a constant of all somatic cells, from the RNA/DNA relationship it is possible to weigh the cells' proteic synthesis.

Based on such objective documents, it is possible to formulate an interpretation of the histochemical meaning that, given our present knowledge, must be given to the phenomenon of tissue fluoroscence. It seems that the different colouring metachromatic behavior of the two nucleic acids is due to the fact that in tissues the polymerization degree of the desossiribonucleic acid is greater than the ribonucleic one. If, in fact, the DNA is depolymerized by treatment with enzymes or acids, the nuclei take on a sharp red fluorescence after colouring with acridine orange; furthermore, the cell elements containing RNA lose their strong absorption to the U.V. at 2650 Å after treatment with ribonucleasis.

From experimental inquiries and on the basis of some formulated hypotheses, we can make some interpretations on the interaction between fluorochrome and nucleic acids and on the different fluorochromization of DNA and RNA. In fact, it is believed that the live fluorochromization is determined by the action of the basic fluorochrome on free acid-phosphoric groups through a displacing action that it exercises on proteins normally less basic and, however, well-plugged to the abbient pH. Presently, the most realistic assumption seems to be that of a mostly electrostatic interaction with the nuclear DNA and of an absorption with the cyto-

plasmatic and nucleolar RNA. In fact, tied proteins, more or less replaceable, participate in the RNA with isoelectrical points higher than the nuclear ones: therefore, a greater quantity of colouring concentrates on the RNA rather than on the DNA.

The red cytoplasmatic and nucleolar colouring would be due to a complex of absorption of colouring in the ribonucleoproteins with the formation of dymers and polymers.

Concerning the metachromatic behavior of acridine orange at tissue and cell level, it is well known how much importance is generally given the determination, even approximate, of the chromotrope power: in fact, even with a small magnification, the microfluoroscopic pictures of the organs and the embryonal tissues examined, very active physiologically and with a high metabolism, show an intense polychromatic fluorescence of the nucleo-cytoplasmatic structures. Furthermore, at higher enlargements, the morphology is easily distinguished, not only for the sharp contrast in color between the nuclear and the cytoplasmatic fluorescences, but also for the clearly visible cell structures, which are not altered, since the fluorochrome is employed at a high dilution rate (1 : 10.000).

Therefore the fluorochromization mechanism with acridine orange, tied to the nucleoproteidic metabolism, is of particular interest if we consider the importance that nucleir acids have in the reproduction and development of cells. Undoubtedly the histochemical reaction gives an indication of the many cell activities, as well as the biological attitude already in the first phases of the development of organs, and constitutes a precious connection for the study of biochemical characteristics of embryonal tissues, signs of a high trophysm and a lively functional activity.

Such evaluation, as has been made clear in the description of the results obtained, allows us to better focus, both at a nuclear level and at a cytoplasmatic level, on fundamental vital processes of the cells tied to the activities of proteic syntheses.

REFERENCES

1) McRobbie D. W., Hancock A. P., Castellano I. A.: "A figure of merit for the assessment of image intensifier systems". *Br. J. Radiol.*, 1992, *65,* 878.
2) Wessel G. M., McClay D. R.: "Two embryonic tissue-specific molecules identified by a double label immunofluorescence thechnique for monoclonal antibodies". *J. Histochem Cytochem,* 1986, *34,* 703.
3) Holzgreve W., Garritsen H. S., Ganshirt-Ahlert D.: "Fetal cells in the maternal circulation". *J. Reprod. Med.,* 1992, *37,* 410.
4) Wollff D. J., Schwartz S.: "Characterization of Robertsoniantranslocations by using fluorescence in situ hybridization". *Am. J. Hum. Genet.,* 1992, *50,* 174.
5) Hossmann K. A., Linn F., Okada Y.: "Bioluminescence and fluoroscopic imaging of tissues pH and metabolites in experimental broin tumors of cat". *NMR Biomed.,* 1992, *5,* 259.
6) Rudin S., Bednarek D. R.: "Region of interest fluoroscopy". *Med. Phys.,* 1992, *19,* 1183.
7) Sani G., Citti U., Caramazza G.: "La microscopia di fluorescenza nella citodiagnosi del cancro". Ed. Cappelli, Bologna, 1963.

Address reprints requests to:
M. PELLICANO
Riviera di Chiaia, 118
80122 Napoli (Italy)