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Experimental Hyaline Droplets in the Rat Adrenal Cortex

Immunohistochemical and Enzyme Histochemical Studies

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Experimentelle hyaline Tropfenbildung in der Nebennierenrinde der Ratte

Immunhistochemische und enzymhistochemische Untersuchungen

Zusammenfassung. Durch Verabreichung von Methylandrostenediol entstanden hyaline Tröpfchen und eiweißhaltige Vacuolen in Nebennierenrindenzellen bei weiblichen weißen Ratten. Die cytoplasmatischen Ablagerungen zeigten keine positive alkalische Phosphatasereaktion bei entsprechenden Färbungen, jedoch eine positive Farbreaktion bei saurer Phosphatase und unspezifischer Esterase. Die dichteren und regelmäßigeren Tröpfchen wiesen eine gleichmäßigere und kräftigere Fermentaktivität auf. Dagegen enthielten die vacuolären Ablagerungen wasserhaltige Substanzen. Die als frischer abgefaßte Ablagerungen war enauf saure Hydrolasen schwach positiv. Mit immunohistologischen Methoden konnte man nachweisen, daß sie homologes Plasmaeiweiß enthielten. In den meisten Vacuolen fanden sich ausschließlich Albumine, einige enthielten jedoch auch Globuline und Fibrin in unterschiedlicher Menge Die gleichzeitige Verwendung von verschiedenen, mit FITC und LRB 200 markierten Antikörpern erbrachte den Nachweis, daß die Tröpfchen aus einer Plasma-Eiweiß-Mischung bestanden. Durch die immunohistologische Methode wurde deutlich gemacht, daß die Tröpfchen nicht homogen sind. Man darf annehmen, daß die intracellulären „hyalinen" Ablagerungen eine gewöhnliche Reaktion der Nebennierenrindenzellen darstellt, die mit den Eiweiß-resorption-Tröpfchen, Phagosomen und deren weiteren Entwicklungsstadien wie sie in anderen Organen vorkommen, vergleichbar sind. So werden diese Gebilde mit dem pinocytär-lysosomalen System der Nebennierenrindenzelle in Bezug gebracht.

Summary. Hyaline droplets and protein-containing vacuoles were produced in adrenocortical cells of female albino rats by methylandrostenediol administration. The lesions were shown to be invariably negative with the method for alkaline phosphatase, but they showed a varying degree of positive staining with the reactions for acid phosphatase and nonspecific esterases. The denser and more regular "droplets" tended to exhibit a more regular and stronger enzymatic activity, whereas the lesions of the "vacuole" type containing a less dense, presumably watery material; those regarded as "younger" were only faintly, if ever, positive with the methods for acid hydrolases.

With the immunohistochemical method the authors were able to demonstrate the presence of homologous plasma proteins within the lesions. Most of the droplets and vacuoles were shown to contain albumin, and some of them could be demonstrated to contain also globulin and fibrin in varying amounts. The simultaneous use of different antibodies labelled with contrasting fluorescent dyes as fluorescein isothiocyanate and lissamine rhodamine B 200, disclosed that most droplets were composed of a mixture of plasma proteins. With that method, it was possible to visualise inhomogeneities in the composition of droplets which largely corresponded to those described previously in preparations stained with conventional histological techniques as well as with some histochemical methods.

The authors conclude that this type of intracellular "hyaline" deposit represents a rather common reaction of adrenocortical cells and that it is comparable to the protein absorption droplets, phagosomes and their subsequent developmental stages known in other organs, thus being related to the pinocytotic-lysosomal system of the adrenocortical cell.

The problem of hyaline droplet formation within the adrenocortical cells has been discussed in considerable detail in several previous papers of ours devoted, in part, to hyaline droplet formation within the adrenal gland of human beings and, in part, to experimental hyaline droplet production in the rat (MOTLÍK and JANOUŠKOVÁ, 1960, 1963).

From the *morphological* point of view, we have stressed the differences of hyaline droplets in size and shape, as well as differences in their affinity to certain stains. Some "droplets" were shown to be of a rather irregular shape, more-or-less resembling "lakes" of hyaline material, but such formations were shown to exhibit gradual transitions to ordinary rounded intracytoplasmic hyaline bodies. The same is true of the so-called "protein-containing vacuoles" (PCV), intracytoplasmic vacuoles containing mostly irregular clots of hyaline material which was frequently shown to assume the shape of crescents closely adherent to the walls of the vacuoles (confront with the "crescent-shaped bodies" of SELYE; SELYE and SALGADO) and to show, too, gradual transitions to true hyaline droplets of conventional morphological appearance.

The review of the literature as well as the findings of ours have led us to the preliminary conclusion that adrenocortical HD represent a lesion of variegated etiology and pathogenesis. However, a large proportion of them were believed to represent a pathobiotic lesion presumably associated with an intake of some proteinaceous material into the cell rather than representing a process of secretion or discharge. Furthermore, some of the morphological as well as histochemical findings indicated that the material ingested could have originated from the blood plasma and that the adrenocortical HD formation could be possibly regarded as secondary to some phenomena accompanying increased adrenocortical stimulation, particularly increased capillary permeability rather than related to the cellular secretory activity as such. The following investigations were undertaken to check this presumption.

Material and Methods

The experiment was carried out with the aim to demonstrate the presence of some enzymes and of plasma proteins within the experimental adrenocortical lesions of rats subjected to methylandrostenediol (MAD) or MAD + somatotropin (STH) administration, known to produce these lesions regularly in sufficient amounts (MOTLÍK and JANOUŠKOVÁ, see also for further literature).

Twenty-four female albino rats of the Wistar strain (Experiment V-66) whose average weight was about 130 g at the beginning of the experiment were used. The experiment lasted for 60 days. The rats were divided into groups consisting of 4 to 5 animals each. The controls (Group I, 5 animals) received no treatment. The experimental animals (Groups II—IV, 14 animals) were subjected to a left-sided nephrectomy performed by a semi-sterile technique. Since the other day after the operation, the animals were given 1 per cent saline solution for drinking in stead of water. Since the third day after the operation, the animals received daily subcutaneous injections of 10 mg of MAD (Spofa, 0.2 ml of microcrystalline suspension) the hyaline droplet producing potency of which had been verified by our previous experiments. Its efficiency was checked during the whole course of the experiment by weighing the animals. The last group of experimental animals (Group V, 5 animals) differed from the MAD-groups in that the former received approximately 6 E. U. of STH per animal per day as a subcutaneous injection of 0.2 ml of Somatotropin Spofa known to aggravate the MAD-

induced adrenocortical lesions in the rat. Its effectiveness was also followed by weighing the animals throughout the experiment.

At the end of the experiment, i. e. 57 days after the commencement of MAD (or MAD and STH) administration, the animals were sacrificed by decapitation following a head blow. Their adrenals were dissected from the surrounding fat. The left adrenal glands of two control animals and of fourteen experimental ones (randomly selected from all groups) were quick-frozen on metal microtome holders by immersion into petroleum ether precooled to approx. 80° C in the mixture of acetone and dry ice (TOBIE), blotted from excess of petroleum ether and wrapped tightly into a polystyrene foil. The material was kept on dry ice until the moment of further processing, which took place within three weeks after the termination of the experiment. The right adrenal glands of all the experimental and control animals were fixed in cold neutral calcium-formalin overnight, thoroughly washed in cold distilled water and rapidly embedded into paraffin by the vacuum technique, so that the embedding process was finished within two hours. The temperature did not exceed 56° C. Adrenal glands of two controls and 6 experimental animals were processed for electron microscopic studies, the results of which will be reported separately. Several experimental and control adrenals were sectioned on the freezing microtome for the purposes of lipid and enzyme histochemistry.

Deparaffinised sections of the paraffin-embedded tissue blocks were stained with haematoxylin and eosin and with many other histological stains. The histochemical reactions performed with the paraffin-embedded material included the PAS-reaction and reactions for some hydrolytic enzymes, namely the alkaline phosphatase (AP), acid phosphatase (AcP), and nonspecific esterases (NSE) using a-naphtyl-acetate (aE) and naphtyl-AS phosphate (ASE) as substrates. The couplers used were either Fast Red ITR or Fast Blue RR for AP, hexazotised pararosanilin for AcP, and Fast Blue RR and Fast Blue B, respectively, for the ASE and αE. The incubation times were 10 min for AP, 2 hrs for AcP, and 10—20 min for NSE. The same reactions were also carried out in frozen sections. For the details of the paraffin-enzyme technique used see the paper of MÜLLER.

The immunohistochemical reagents used in this study were prepared from sera of rabbits immunised with rat plasma fractions. The following fractions were used as antigens: Fibrinogen obtained by the method of WARE, albumin obtained by means of preparative electrophoresis in agar gel, and globulin (fraction gamma$_2$) precipitated from rat sera with half-saturated ammonium sulphate solution and purified on a DEAE-cellulose column (LOSPALUTTO et al.).

Rabbits were immunised with a total of 20—25 mg of protein antigen emulsified with complete Freund's adjuvant and injected intramuscularly twice at weekly intervals followed by three intravenous injections of antigen without adjuvant given every other day. After two weeks the animals' sera were examined for their antibody content by means of double diffusion in agar gel and by immunoelectrophoresis.

The animals were bled from the jugular vein under nembutal anaesthesia. The globulin fractions of immune sera were obtained by precipitation with half-saturated ammonium sulphate after prolonged dialysis, each of these globulin fractions was labelled with fluorescein isothiocyanate (FITC) and Lissamine Rhodamine B 200 (LRB), respectively. The labelling procedure with FITC was performed according to the method of MARSHALL et al. using 0.05 mg of fluorochrome per 1 mg of protein as a rule. Labelling with LRB followed the original description of CHADWICK et al. with minor modifications. Excess fluorochrome was removed by overnight dialysis followed by gel filtration on Sephadex G 50 (ZWAAN and DAM).

All the histochemical reagents were checked by double diffusion in agar gel and by immunoelectrophoresis in a reaction with the antigen used for immunisation and the rat serum. The trace amounts of "unwanted" antibodies occurring probably as the result of the presence of other serum fractions in antigens used for immunisation were removed by precipitation. The anti-fibrin reagent was rendered monospecific after absorption with rat serum previously absorbed with bentonite (NIEWIAROWSKI). The anti-albumin reagent was depleted of anti-globulin antibodies by absorption with small amounts of gamma$_2$-globulin. The purified reagents were finally characterised as to their FITC or LRB and protein content. From these data the FITC: protein ratio was calculated, the optimal dilution for staining being selected from the results of trial staining of serial sections of the rat spleen.

The immunohistochemical reagents thus prepared and characterised were quick-frozen in small aliquots and stored at — 22° C until use. Before use they were absorbed with 50 mg/ml of acetone-dried mouse liver powder.

Frozen tissue blocks were sectioned in the cryostat at — 18° C. Sections approximately 6 micra thick were thawed on microscope slid es and air-dried at room temperature for 20 minutes. They were then fixed in absolute acetone for 10 minutes and incubated with two drops of the absorbed and appropriately diluted immunohistochemical reagent for 45 minutes in a moist chamber at room temperature. The sections were then carefully washed in three changes of buffered (pH 7.6) physiological saline and finally mounted in buffered (pH 7.6) glycerine under cover slips. Double staining reactions were carried out with the LRB-labelled reagent used first, followed by washing in buffered saline for 30 minutes and subsequent staining with FITC-labelled reagent. Nine consecutive serial sections from each adrenal gland were examined as a rule. They were divided into three sets consisting of three sections each. Each set was stained first with the FITC-labelled reagent, one section was then mounted and the remaining two were stained with the LRB-labelled reagents.

The specificity of immunological reactions was ascertained by control procedures consisting in staining the adrenal glands of untreated rats and those of the experimental ones with fluorescent-labelled heterologous reagents as e. g. anti-human globulin, as well as in staining with homologous reagents after incubation of the sections in unlabelled homologous reagents (specific inhibition).

A Reichert "Zetopan" microscope equipped with a HBO 200 mercury lamp, UG 1/1.5 mm exciter filter, GG 9/1 mm barrier filter and dark field condenser was used throughout this study. Black and white microphotographs were taken on Ilford XPX film, colour diapositives were made on "Kodak Ektachrome High Speed 135" film with exposure times ranging from 1 to 3 minutes.

Results

A. With the *routine histological stains* the results corresponded to those reported in the previous papers of ours (cf. MOTLÍK and JANOUŠKOVÁ). Hyaline droplets and "lakes" of hyaline material as well as PCV could be demonstrated in all experimental animals. Some of them also contained PCV with a crescent-shaped hyaline contents. The results of Sudan staining and of the PAS-reaction as well as of the other staining methods used were also identical with those reported in the previous paper of ourselves. Various types of tinctorial inhomogeneity including the presence of fibres could be also disclosed in some HD and "lakes" of hyaline material. Occasional nucleated cells probably of haematogenic origin and occasional red blood cells (particularly at sites of haemorrhage) could be disclosed within the adrenocortical cells. Numerous small cells with dense nuclei containing various amounts of tiny eosinophilic and PAS-positive granules were found intercalated between activated adrenocortical cells within the inner fasciculata and the reticularis.

B. The results of the *histochemical methods for the hydrolases* were substantially identical in the frozen as well as in the paraffin material. They differed only in that there was some loss of diffuse plasmatic staining with the AP, AcP, ASE and particularly with the alpha-E method. As far as the intracytoplasmic "hyaline" formations are concerned, the results were entirely identical in the paraffin and in the frozen sections so that they can be discussed together.

With the *AP method* (Fig. 1), all the intracytoplasmic structures in question were completely negative. The reaction product was confined to the capillary walls, especially to those of the capsule, of the glomerulosa and of the outer fasciculata. The reaction in the capillary walls was much weaker but still slightly

positive in the inner fasciculata and reticularis. No substantial differences were noted between the experimental animals and the control ones except for a slight increase in diffuse plasmatic staining and cell membrane-bound enzyme activity in the activated cells of the inner and middle fasciculata in the MAD-treated animals.

With the method for *AcP*, some of the intracytoplasmic structures studied were found to be positive to a different degree, however, some of them were completely negative. The PCV of all types tended to be either negative or slightly

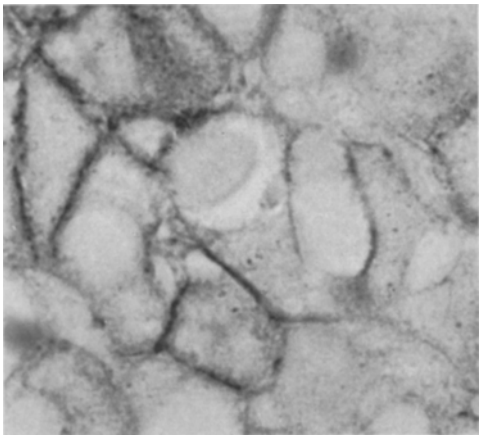


Fig. 1. Adrenal cortex of experimental animal. Alkaline phosphatase method. Some enzyme activity can be seen at the cell membranes, but there is a completely negative hyaline droplet in the centre of the field. Alkaline phosphatase, azo-coupling method, no counterstain. Approx. $\times 900$

positive, whereas the result of the reaction in the HD and in the "lakes" ranged from complete negativity to rather strong positivity, usually strikingly contrasting with the faintly positive or almost negative cytoplasmic backgroundl The small cells containing cytoplasmic granules seen within the inner cortica. layers showed a moderate granular and diffuse cytoplasmic staining with this reaction. There were also some AcP positive granules in the (otherwise intact) cells of the zona reticularis and in those of the juxtamedullary zone as well as in the adrenocortical cells within the medulla. These granules could be also seen in the corresponding cells of the control adrenal glands, and they were shown to correspond to the normally occurring hyaline acidophilic granules seen in the routine stained slides. The Golgi regions of the adrenocortical cells were also positive with this reaction. This was most marked in the activated fasciculata cells.

With the reactions for *NSE*, the results were very similar with those obtained with the reaction for AcP, (Figs. 2 and 3). In general, the reaction was somewhat stronger and thus the picture was more contrast-rich than with the AcP method. There were also large differences in the positivity of the reaction in the various formations under discussion, and there was also a tendency to a weak-to-negative reaction in the PCV (Fig. 2). A wide range of positivity and an occasional negative result was seen with the HD and the "lakes". The small granulated intercalated

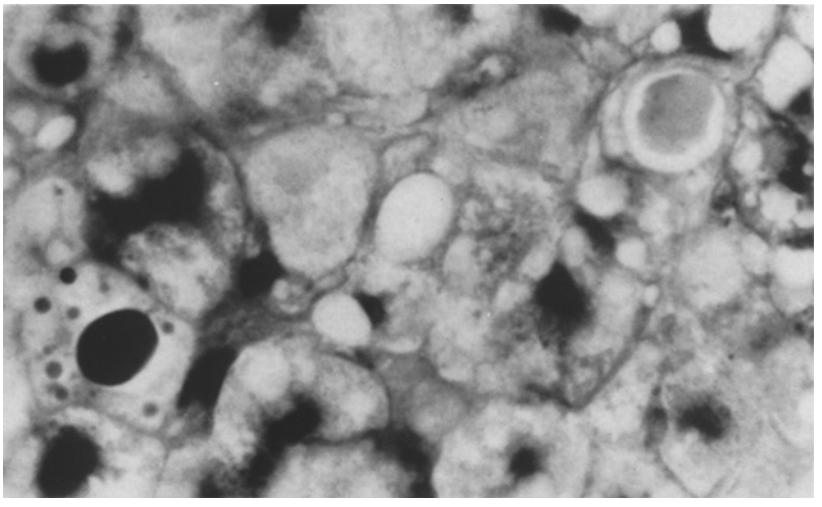
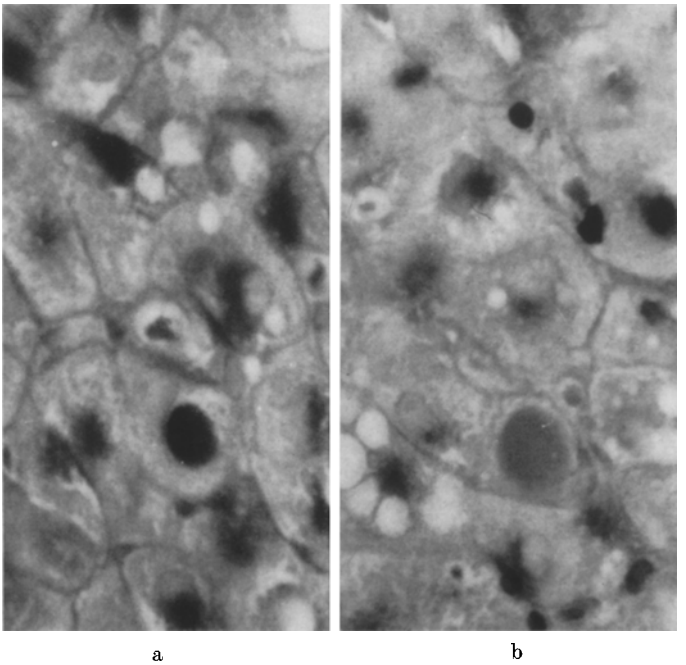


Fig. 2. Adrenal cortex of experimental animal. Method for naphtyl-AS esterase, no counterstain. Note the different degree of enzyme activity of the multiple droplets present in the adrenocortical cell in the left lower corner. Note another, almost negative formation of somewhat irregular shape in the right upper corner of the figure. Strong activity can be also seen in the Golgi regions of the adrenocortical cells. Approx. ×900



a b

Fig. 3a u. b. Adrenal cortex of experimental animal. Method for α-naphtyl esterase, no counterstain. a A strongly positive droplet can be seen in the lower half of the figure. Note positivity of Golgi regions. b A small, strongly positive droplet at the upper edge of the figure. A huge, almost negative droplet in its lower half. Perinuclear regions positive. Approx. ×900

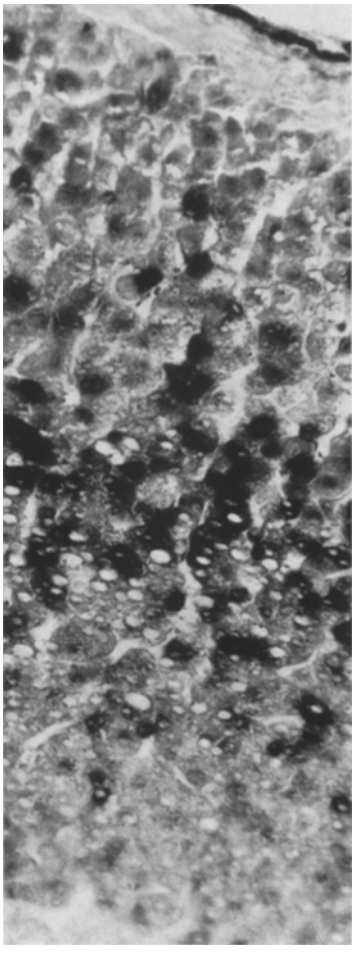


Fig. 4

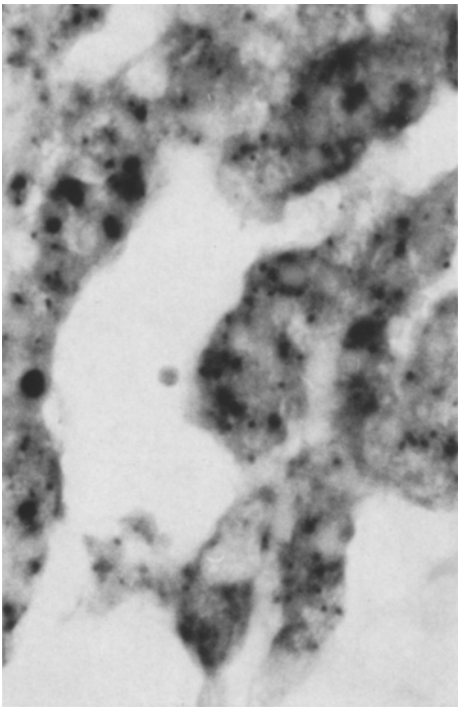


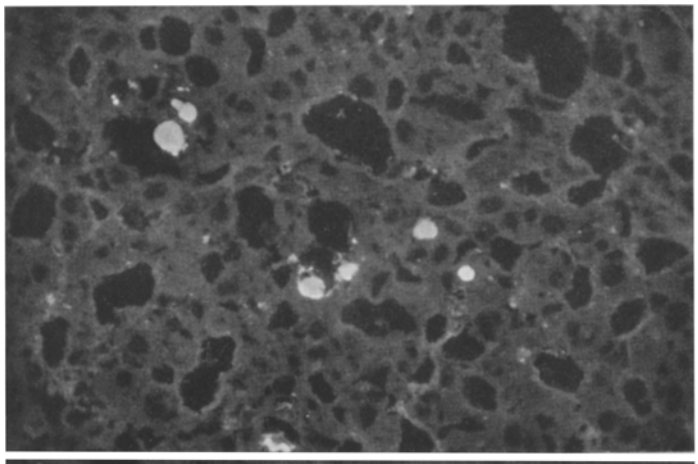
Fig. 5. Zona reticularis of control animal. Small granules can be seen in all cells. In some of them, larger, droplet-like formations occur (left). Negative medulla in the right lower corner. Method for naphtyl-AS esterase, no counterstain. Approx. ×900

Fig. 4. Adrenal cortex of experimental animal. Note the distribution of alpha-naphtyl esterase positivity with its maximum at the border between the vacuolated inner portion and the nonvacuolated outer portion of fasciculata. Approx. ×140

cells of the inner cortical layers and the granules present in the experimental and control adrenocortical cells of the inner fasciculata, reticularis and of the "central" cortex were also strongly positive with this method.

The esterase-positive cells were concentrated in an ill-defined band at the boundary between the vacuolated and the nonvacuolated cortical areas, i. e. at the level of the inner fasciculata (Fig. 4). However, isolated strongly positive cells were found scattered in all cortical layers. Some of them contained esterase-positive HD, which unfortunately could not be adequately visualised within their strongly positive cytoplasm.

C. Immunohistochemical Studies. In unstained cryostat sections examined in ultraviolet light several grey-bluish autofluorescent intracytoplasmic droplets were readily visible. In serial sections from the same tissue blocks stained with the PAS-method, these autofluorescent droplets were identified with the HD described formerly.



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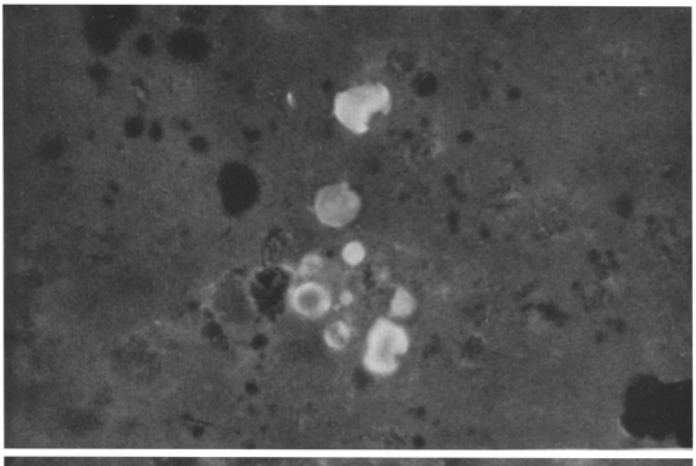

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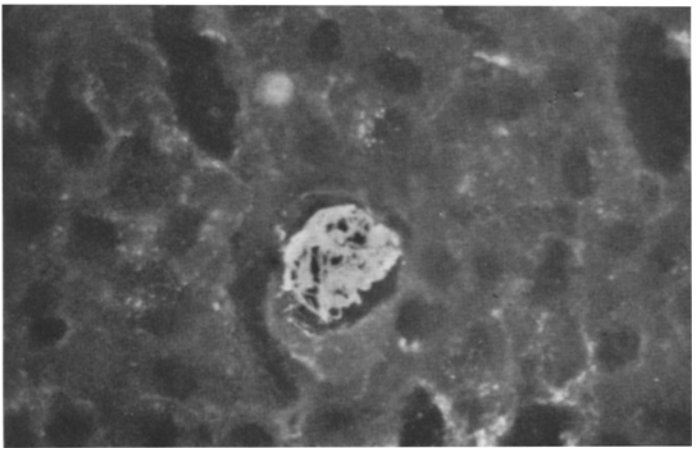

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Fig. 6*. Several brightly fluorescent droplets of various size and shape in adrenocortical cells. FITC-labelled anti-albumin antibody

* Figs. 6—11 have been taken from adrenal cortices of experimental animals. Fluorescent antibody method, for details see the text. Original colour transparencies reproduced black and white

In sections stained with the *anti-albumin* reagent (Fig. 6) all hyaline droplets present in a given section fluoresced brilliantly. Their fluorescence pattern was usually homogeneous and of uniformly high intensity. In some cells, however, irregularly shaped or circular areas of markedly lower intensity of specific fluorescence were seen. They were usually situated in the central parts of the HD. Almost every section contained droplets showing triangular or crescent-shaped areas at the periphery almost completely devoid of specific fluorescence.

In sections stained with the *anti-globulin* reagent (Fig. 7), the vast majority of HD showed a very intense specific fluorescence, however, in almost every section single autofluorescent (unstained) droplets could be found, as well as droplets exhibiting uniform fluorescence of low intensity. In rare instances the intense specific fluorescence was limited to the droplet periphery forming a brilliant annulus contrasting distinctly with the dimly fluorescing core of the droplet. In still other droplets conglomerates of tiny fluorescent granules were discernible and very few droplets were seemingly composed of several globules.

The staining with the *anti-fibrin* reagent (Fig. 8) revealed the presence of this protein in the majority of HD in the material examined, however, few autofluorescent (unstained) droplets were also encountered. The fluorescence of fibrin was usually of uniform intensity and distribution, in many droplets, however, a more brilliantly fluorescent core was observed. Among all these rounded, usually circular or oval formations, there also occurred large, irregularly shaped droplets exhibiting specific fluorescence of medium intensity. In these sections and in sections of control adrenals stained with the anti-fibrin reagent, a very intense specific fluorescence of an endothelial film was noted. However, large masses of intravascular fibrin were encountered only in the venous sinuses of the adrenals of the experimental group.

The staining with *two different reagents labelled in contrasting colours* disclosed the presence of various plasma proteins within the same hyaline formations as well as a great variability of the spacial and quantitative interrelations of their different protein components (Figs. 9—11). The most striking example of such a complexity was offered by adrenal cells containing multiple HD in their cytoplasm (Figs. 9 and 11). These droplets were usually all of one colour, either pure yellow-green or orange-red, or they exhibited a rich "mixed" yellow fluorescence. In a significant percentage of cells, however, some droplets exhibited "mixed" yellow fluorescence, some others were orange-red and still other ones fluoresced yellow-green. Very rarely HD showing a distinct spacial distribution of yellow-green and orange-red fluorescence were found.

In sections treated with the LRB-labelled anti-albumin reagent and counterstained with the FITC-labelled either anti-globulin or anti-fibrin reagent, the majority of HD fluoresced deep orange, which is believed to reflect the prevalence of their serum albumin content. The staining with LRB-labelled anti-globulin

Fig. 7. A group of droplets showing intense specific fluorescence. Note some inhomogeneity of fluorescence, namely a negative core in one of the droplets, and somewhat irregular outlines of others. FITC-labelled anti-globulin antibody

Fig. 8. A vacuole-like formation containing a clump of fibrillar material, exhibiting an intense specific fluorescence. FITC-labelled anti-fibrin antibody

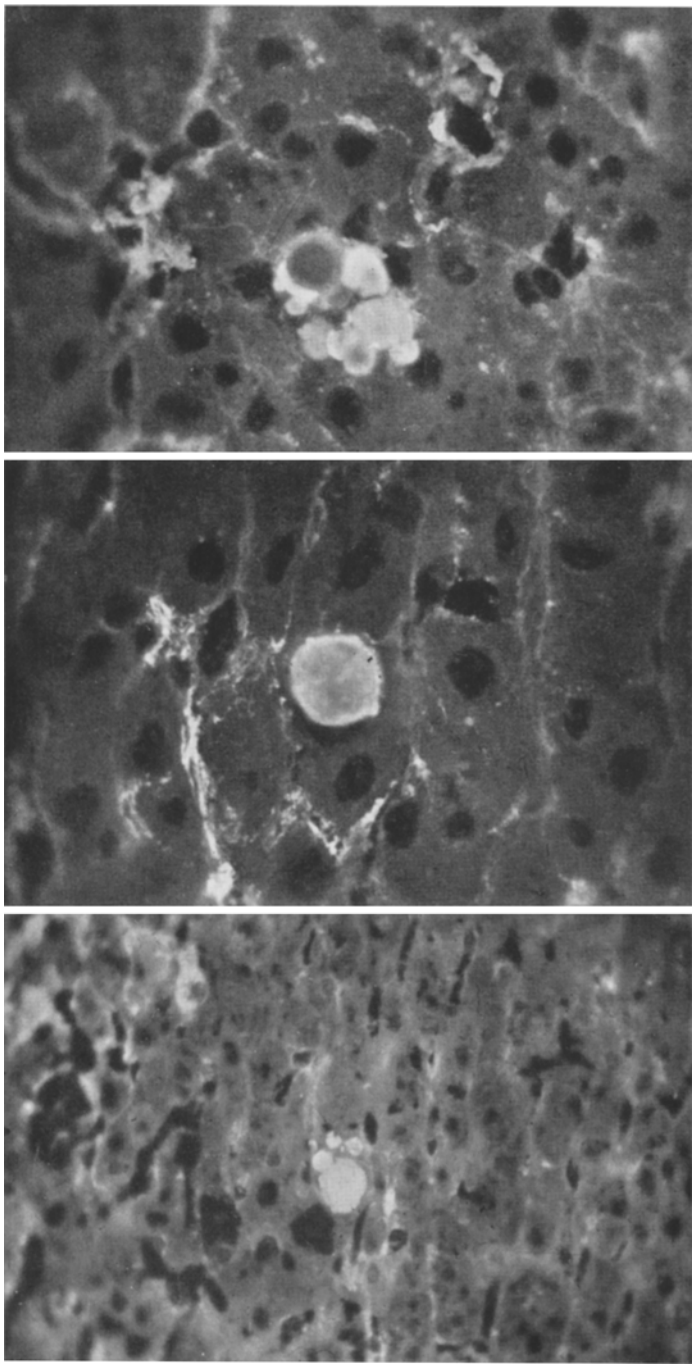

Fig. 9. A group of droplets showing mostly the "mixed" type of fluorescence, however, of a varying hue indicating differences in the ratio of participating proteins. Note an almost negative droplet showing only a dim red fluorescence at its periphery. Double staining method: LRB-labelled anti-albumin antibody + FITC-labelled anti-globulin antibody

reagent and FITC-labelled anti-fibrin reagent resulted in a "mixed" rich yellow fluorescence of the majority of HD, usually without any apparent predominance of either constituent. In all the adrenals examined HD were found which, notwithstanding the combination of reagents used, fluoresced in one colour. Most numerous droplets of this kind were revealed by the anti-albumin reagent, those revealed by the anti-globulin reagent were distinctly less numerous as were those stained by the anti-fibrin reagent alone. The most conspicuous feature encountered in sections stained with the anti-albumin reagent and supplemented with the anti-globulin reagent or the anti-fibrin one concerned the spacial distribution of the plasma proteins within a single HD. In these droplets the orange-red fluorescence of albumin occupying the central part was surrounded by a rim of yellow fluorescence of globulin or fibrin. Rarely, the crescent-shaped area of albumin fluorescence in the intermediate part of a droplet was partially obscured by the "flare" of brilliant fluorescence of globulin or fibrin.

The control procedures confirmed the specificity of the immunofluorescent staining. No fluorescence was observed in sections treated with heterologous reagents and in sections from adrenals of untreated rats. There was a distinct inhibition of specific fluorescence in sections pre-treated with unlabelled reagents.

Discussion

The lesions produced in the present experiment were virtually identical with those obtained in our previous ones (MOTLÍK and JANOUŠKOVÁ).

The histochemical findings as presented in the present paper have shown that the lesions under discussion were in part moderately to strongly positive for the activity of acid hydrolases. This particularly applies to the hyaline lesions, namely to the HD and the "lakes", the vast majority of which were positive for AcP and NSE. In contrast, the PCV were mostly negative or faintly, if ever, positive for these enzyme activities. On the other hand, there seems to be no fundamental difference in the plasma protein content of these intracytoplasmic lesions demonstrable by the immunofluorescent technique, as employed in our study, which, however, did not permit us to correlate the results of conventional histological methods with those of the enzyme histochemical and the immunohistochemical ones within the same droplets, i. e. in parallel or identical sections.

As we have already pointed out in the previous papers of ours, lesions very similar to those discussed here were shown to occur also in other organs in humans as well as in experimental animals under various conditions. Among those most strikingly similar are the lesions occurring in the liver, known either as the "asphyctic vacuoles" of KETTLER or the "coacervates" of ALTMANN, as the

Fig. 10. A large droplet-like lesion showing the "mixed" type of specific fluorescence as well as some staining inhomogeneity of its interior. Remains of fibrin can be seen at the walls of the capillaries in this section. Double-staining method: LRB-labelled anti-albumin antibody, + FITC-labelled anti-fibrin antibody

Fig. 11. Several droplets within the cytoplasm of an adrenocortical cell. Some of them show the "mixed" type of fluorescence with a striking prevalence of the red component, a small droplet at the upper edge of the group exhibits an almost pure green specific fluorescence. Double-staining method: LRB-labelled anti-globulin antibody + FITC-labelled anti-fibrin antibody

"vacuoles" or "acidophilic bodies" found in the liver cells in experimental asphyxia, carbon tetrachloride intoxication or renin administration (e. g. Nairn et al.).

In the past, many different methods were used to demonstrate the presence of plasma proteins within such lesions. Evans blue and various other dyes were used for this purpose by some authors (e. g. Doniach and Weinbren, Aterman and others). Ericsson used homologous haemoglobin as a tracer of the exogeneous material ingested into the (renal tubular) cells, and Straus et al. used horseradish peroxidase for the same purpose. Nairn and his associates employed the direct fluorescent protein tracer method to demonstrate the incorporation of plasma proteins into the cytoplasm of liver cells in carbon tetrachloride intoxication and some other states.

Our suspicion that the adrenocortical HD and similar lesions might probably contain proteinaceous material ingested from the blood stream has been primarily based on the histochemical similarity of the intracellular deposits with the material present in the neighbouring vessels and in the extravasates, as well as on the presence of fibres resembling fibrin within the droplets, and by the finding of red blood cells and of probably haematogenic nucleated cells within the intracellular lesions (Motlík and Janoušková). However, the most exact and most specific way of demonstrating the presence of proteins within these lesions and of analysing their character and provenience is the immunohistochemical method. This has not been used so far for the demonstration of plasmatic proteins within intracytoplasmic lesions of this type in the adrenal cortex.

Until recently, Nairn and his associates have been the only authors who have briefly mentioned the presence of plasma protein-containing droplet-like lesions within the adrenocortical cells of a rabbit intoxicated with carbon tetrachloride, which was an incidental finding in their experiments. Their studies, however, were carried out with the fluorescent direct protein tracer method (Nairn and ass.) where the tracer protein was administered 24—48 hrs after carbon tetrachloride administration. We believe that the character of the lesions described in the present paper is much the same as that of the lesions observed by Nairn and his co-workers, though its causal pathogenesis is probably different. However, in both cases an increase in vascular permeability is strongly suspected to play an important role in the development of these intracytoplasmic deposits.

As far as the composition of the droplets is concerned, the studies of ours have clearly demonstrated that the droplets in question consist of a mixture of plasma proteins. All droplets seemed to contain albumin, whereas the remaining two plasma fractions participated in their composition to a varying degree. With various antibodies, occasional droplets were shown to be inhomogeneous, showing unstained foci of different shape. This could be due to an inhomogeneous mixture of proteins, but an occasional admixture of cells to the PCV or HD contents could not be excluded with certainty. This inhomogeneous fluorescence is believed to correspond — to a certain degree — to the staining inhomogeneity observed with different histological stains and histochemical reactions. The staining inhomogeneity of the vacuolar lesions and of the hyaline deposits as observed within the cytoplasm of liver cells in carbon tetrachloride intoxication by Nairn et al. using the direct fluorescent protein tracer method should be evaluated with caution, since the non-fluorescent remainder of the lesions showing a fluorescent

rim or "crescent" at their periphery might have also contained plasma protein which, however, could have escaped from the vessels during the period between the moment of carbon tetrachloride administration and that of the injection of labelled protein. This is not the case if the immunofluorescent method is used, and therefore the inhomogeneities observed with this method reflect the true pattern of plasma fractions within the lesions.

With the anti-fibrin antibodies, occasional droplets were shown to contain fibrillar material as shown in Fig. 8. Though this material could not be directly identified with that visualised by various histological methods, namely the "fibrin stains", for technical reasons, this finding largely supports the presumption that the fibrillar material observed in conventional preparations really represents fibrin.

The increase in the diffuse cytoplasmic activity of acid hydrolases, particularly of the nonspecific esterases, most marked at the boundary between the vacuolated (inner) and the nonvacuolated (outer) part of the cortex was a striking feature of the adrenal cortices of all animals receiving MAD. It should be reemphasized that it is this region where most of the small granulated cells with pycnotic nuclei are found and that this is also one of the sites where hyaline droplets (usually of the "mature", i. e. dense and regular type) occur most frequently. It must be admitted that all these changes might represent signs of adrenocortical degeneration associated with autophagia, the small cells being, in fact, dying elements. This, of course, requires verification by electron microscopy. However, these changes could be the base of the well known fact that, in spite of obvious adrenocortical activation, the weight of the adrenals does not change substantially after MAD administration and that the administration of MAD interferes with ACTH-induced adrenocortical hyperplasia (SELYE and HEUSER).

The majority of the tiny "HD" scattered throughout the inner zones of the adrenal cortices of both the experimental and control animals which are strongly positive for the acid hydrolases examined, are believed to represent lysosomes.

It is to be noted that besides the HD (and related formations) in the experimental animals, no corpuscular intracytoplasmic specific fluorescence could be noted in any of the experimental or control animals. No counterpart to the increase in the cytoplasmic staining of adrenocortical cells with the methods for acid hydrolases could be disclosed with the immunofluorescent technique. Specific fluorescence was naturally seen within some vessels, and very rarely some diffuse staining of the cell cytoplasm and some intercellular extravascular material could be disclosed. However, the exact localisation of the specific fluorescence of this type in the given material was frequently dubious.

In conclusion, we can say that the recent findings of ours have largely confirmed our previous findings and supported their explanation. We believe that *most of the hyaline droplets and similar lesions* developing in the adrenocortical cells of rats after MAD administration contain ingested material of plasmatic origin and in view of the histochemical findings presented can be characterised as a *cellular dystrophy (pathobiosis) related to the pinocytotic-lysosomal system.* Both the formations under discussion are believed to represent developmental steps of the cytophagic process in accordance with the views of NOVIKOFF, ERICSSON and STRAUS. The "younger" lesions correspond to the pinocytotic vacuoles (phagosomes) containing extracellular aqueous material which, if protein-rich, permits

us to denote such vacuoles as "protein absorption droplets" as known from other organs, especially from the kidneys (Ericsson, Straus). Such protein absorption droplets are also originally negative with the methods for acid hydrolases, but they may gradually become positive, most probably owing to fusion with the lysosomes of the affected cell or by receiving the enzymes from the Golgi vesicles (Novikoff, Ericsson and Straus). Thus the older, or more mature lesions appearing "denser" in conventional preparations are more prone to be positive with the methods for acid hydrolases, and the different degree of enzyme concentration within the droplets after fusion of the "protein absorption droplet" "protein-containing pinocytotic vacuole" or phagosome with the enzyme-rich cell components may be responsible for the different degree of positivity as also observed in the experiments of ourselves. Our finding that the "protein-containing vacuoles" which were previously regarded as the less mature stage of the HD are less positive for the activity of the hydrolytic enzymes examined correlates well with the mentioned theory. The same is true of the fact that the "mature", i. e. dense HD have a larger tendency to be moderately or even strongly positive for the enzyme activities in consideration. It should be noted that the results of these histochemical reactions also correlate well with the different degree of PAS-positivity of the two related lesions in question.

Their physiological or pathophysiological significance, however, remains obscure and requires further research, although the secretory nature of this phenomenon can be disproved with certainty. We continue believing (see Motlík and Janoušková) that the droplets occurring within the adrenal cortex after MAD administration vary as to their nature. This, however, can be only elucidated by further electron microscopic studies, which, at present, are in progress.

The occurrence of hyaline droplets is believed to represent a rather common type of adrenocortical cellular reaction, because very similar lesions can be produced under quite different conditions, as e. g. by Polybrene administration (Carroll, Motlík), by the administration of various hormones, poisons as well as by the induction of hypertensive states, starvation, thirst etc. (for lit. see Motlík and Janoušková). The mutual comparison of these lesions as well as their detailed analysis by further histochemical tests, by the fluorescent tracer methods, immunohistochemical methods and by electron microscopy is the object of our forthcoming investigations.

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