THE PROTEIN NATURE OF ACIDOPHILIC CRYSTALLINE INTRANUCLEAR INCLUSIONS IN THE LIVER AND KIDNEY OF DOGS*

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Thompson, Cook, and Hoey[1] have recently reported the histochemical investigation of acidophilic, crystalline intranuclear inclusions (ACN) in the liver and kidney of 13 dogs. It was demonstrated that the inclusions were not composed of any of the following substances: hemoglobin or any of its iron-containing derivatives, minerals, lipids, deoxyribonucleic acid, ribonucleic acid, cholesterol, glycogen, mucin, mucopolysaccharides, polysaccharides, glycoproteins or glycolipids. The possible role of pyrimidines, purines and purine end products in the formation of the inclusions was discounted because of their insolubility in alkali, mineral acid or organic solvents. It was postulated that ACN inclusions were formed as a result of altered permeability of the nuclear membrane, which favored intranuclear retention of protein at periods of intensive protein synthesis in the nucleus. The study, herein reported, was undertaken to determine if protein was a component of the inclusions.

MATERIAL AND METHODS

The tissues employed in this investigation were procured from paraffin-embedded sections of formalin or alcohol fixed tissues from 9 of 13 dogs used in the previous study (cases 5 through 13).[1]

Serial sections, 7 to 8 μ in thickness, were prepared from the paraffin blocks in each case.

The theory of staining designed to demonstrate protein groups in tissue sections has been reported by Danielli.[2] He also reported methods for using the tetrazotized benzidine stain and suggested the application of agents to block specific groups, allowing the demonstration of other groups by staining with dinitrofluorobenzene (DNFB).[3] However, the staining procedures for DNFB were developed in this laboratory and are given below.

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Tetrazotized benzidine was used according to the procedure given by Lillie.[4] Benzoyl chloride was utilized at a concentration of 10 per cent (v/v) in pyridine. Treatment in this reagent for one hour was found sufficient to give complete blockage of the DNFB stain.

The DNFB reagent was adapted from Sanger.[5] A solution of 1 gm. of $NaHCO_3$ in 15 ml. of water was mixed with a solution of .84 ml. of DNFB in 30 ml. of ethanol. The resulting DNFB solution, saturated with $NaHCO_3$, was used at room temperature. Excess bicarbonate was allowed to settle, but was not filtered off. Tissues were regularly stained for 30 minutes. To couple the dinitrophenol derivative to a naphthol, and to enhance the color development, the nitro group was reduced with saturated stannous chloride ($SnCl_2$) in 1 N HCl for 10 minutes at room temperature. The amino group produced was diazotized in a nitrous acid solution (50 ml. of water, 1.25 ml. of concentrated HCl, and .5 gm. $NaNO_2$) for 5 minutes at ice bath temperature. This diazotized derivative was then coupled with betanaphthol, or H-acid [8-amino-1-naphthol-3, 6-disulfonic acid, monosodium salt (procured from Eastman Kodak Company, Organic Chemical Division, Rochester, New York)], according to the procedure of Danielli, as given by Lillie.[4]

According to Danielli,[2] DNFB reacts with the tyrosine groups, amino groups, and sulfhydryl groups of proteins. These groups can be blocked by diazotized p-nitroaniline, nitrous acid, and hydrogen peroxide, respectively.[2] In this work, tyrosine was blocked with diazotized aniline. The diazoaniline was prepared as a stock solution. One hundred ml. of water, 40 gm. of ice, and 3.5 ml. of concentrated HCl were mixed and cooled to 0 to 4° C. in an ice bath. After cooling, 1.38 gm. of $NaNO_2$ were dissolved in the solution, and finally 2.6 gm. of aniline hydrochloride were added with stirring. The stock solution was stored in the refrigerator, and diluted 1:30 with .1 N veronal buffer, pH 9, just prior to use. The sections were treated at ice bath temperature for 30 minutes. The procedure for blocking the amino groups in a tissue section was similar to that used in diazotizing the amino group formed from the dinitrophenol derivative, with the exception that a 15-minute exposure was used to assure complete blockade.

Sulhydryl groups were blocked by treatment with .001 M H_2O_2 at room temperature for 15 minutes.

In the procedures for the specific demonstration of amino, tyrosyl, or sulfhydryl groups, two groups were blocked as above; the section was stained with dinitrofluorobenzene, and this derivative was coupled with H-acid. The stain produced was attributed to the unblocked group.

Staining Schedules

The staining procedure for the demonstration of disulfide linkages is given below. The procedure includes the DNFB staining schedule and coupling with a naphthol (steps 5 through 13).

1. Bring slide to absolute ethanol, remove, and air dry.
2. Place immediately in benzoyl chloride reagent at room temperature for one hour.
3. Rinse with absolute ethanol, take to equilibrium with water, and wash in running water for 5 minutes.
4. Place section in 1 N HCN solution in hood at room temperature for 15 minutes.
5. Wash in running water for 5 minutes.
6. Place in DNFB solution at room temperature for 30 minutes.
7. Rinse in absolute ethanol for 1 minute; ether, 30 seconds; bring back through ethanol to water; wash in running water for 1 minute.
8. Place in saturated stannous chloride in 1 N HCl at room temperature for 10 minutes.
9. Wash in running water for 5 minutes.
10. Place in nitrous acid solution at ice bath temperature for 5 minutes.
11. Equilibrate section in 2 water baths and 3 buffer baths (.1 N veronal, pH 9) at ice bath temperature for 1 minute each.
12. Place in .05 per cent betanaphthol or H-acid solution in .1 N veronal buffer, pH 9, at ice bath temperature for 15 minutes.
13. Wash section in running water for 5 minutes, counterstain with hematoxylin and mount in standard mounting medium.

For the specific demonstration of amino, tyrosyl, or sulfhydryl groups, the groups were blocked individually. By blocking two of the reactive groups, the third could be demonstrated. In order to provide a color comparison, sections which had been treated with all 3 blocking agents and sections which had not been blocked were run. The following schedule includes all 3 blocking agents. To demonstrate a particular group, the blocking reaction for that group was omitted from the procedure.

1. Bring section to equilibrium with water at ice bath temperature.
2. Place section in nitrous acid solution at ice bath temperature for 15 minutes.
3. Rinse section in cold running tap water for 5 minutes, and equilibrate in .1 N veronal buffer, pH 9 at 0 to 4° C.
4. Place section in diazoaniline solution at ice bath temperature for 30 minutes.
5. Wash in cold running tap water for 5 minutes.
6. Place section in .001 M H_2O_2 solution at room temperature for 15 minutes.
7. Wash in running tap water for 5 minutes and proceed with the DNFB staining schedule given in steps 6 through 13 above.

In addition to the procedures given above, liver and kidney sections from cases 5, 6, 10, 11, and 13[1] were subjected to the procedures of Barrnett and Seligman[6-8] for sulfhydryl and disulfide groups, utilizing the 2, 2′-dihydroxy-6, 6′ dinaphthyl disulfide (DDD) stain. Prior to staining for sulfhydryl groups, the deparaffined sections were washed with running tap water for 1½ hours to liberate the sulfhydryl groups.[9] In the disulfide procedure, benzoyl chloride was used to block the sulfhydryl groups.

Results

The results obtained with each staining procedure were sufficiently uniform that the reaction of the acidophilic, crystalline intranuclear (ACN) inclusions can be reported without reference to the tissue or case in which they occurred (Table I). The color characteristics of each stain were as follows:

1. Tetrazotized benzidine. The color range of the ACN inclusions was red to orange, that of the nucleoplasm was blue to violet, and the cytoplasm was orange to orange-brown (Fig. 1).

2. Benzoyl chloride and tetrazotized benzidine. The ACN inclusions were unstained, the nucleoplasm was red to blue, and the cytoplasm was unstained to faint brown (Fig. 2).

Table I

Staining Characteristics of ACN Inclusions

Protein stain	Reaction of inclusions
Tetrazotized benzidine	Positive
Benzoyl chloride and tetrazotized benzidine	Negative
Dinitrofluorobenzene	Positive
Tyrosyl — DNFB	Positive
Amino — DNFB	Weakly positive
Sulfhydryl — DNFB	Positive
Disulfide — DNFB	Negative to weakly positive
Sulfhydryl — DDD	Positive
Disulfide — DDD	Negative

3. Dinitrofluorobenzene (DNFB). The color range of the ACN inclusions was red-brown to mulberry, that of the nucleoplasm was blue to violet, and the cytoplasm was brown (Fig. 3).

4. Tyrosyl groups (DNFB). The color range of the ACN inclusions was indigo to brown, that of the nucleoplasm was purple, and the cytoplasm was light red to tan (Fig. 4).

5. Amino groups (DNFB.) The color range of the ACN inclusions was faint yellow to tan, that of the nucleoplasm was blue to purple, and the cytoplasm was yellow to tan (Fig. 5).

6. Sulfhydryl groups (DFNB). The color range of the ACN inclusions was orange to carmine, that of the nucleoplasm was purple, and the cytoplasm was carmine (Figs. 6 and 7).

7. Disulfide groups (DFNB). The ACN inclusions were usually unstained but occasionally showed a bluish tinge; the nucleoplasm was violet, and the cytoplasm was yellow to tan.

8. Sulfhydryl groups (DDD). The ACN inclusions, nucleoplasm

and cytoplasm were all violet, but the inclusions were more intensely stained (Fig. 8).

9. Disulfide groups (DDD). The ACN inclusions were unstained, the nucleoplasm was faint blue, and the cytoplasm was light yellow.

Discussion

The evidence presented by Thompson, Cook and Hoey[1] indicates that the ACN inclusions do not contain demonstrable carbohydrates, lipids, minerals, or nucleic acids. The results obtained in the present study tend to confirm the previously postulated protein nature of these inclusions. It is thought that the specific reactive groups of protein, which must be in an available form prior to staining,[10] are not appreciably masked by the alcohol or formalin[9,11] fixatives used.

The positive staining with tetrazotized benzidine indicates that the ACN inclusions contain protein or nucleic acid.[3] When tissue sections are blocked by benzoyl chloride and subsequently stained by the same method, only the nucleic acid remains reactive.[4] The ACN inclusions were not stained following treatment with benzoyl chloride. This indicates the absence of nucleic acid[1] and shows that the positive stain of the ACN inclusions with tetrazotized benzidine alone is due to reactive groups normally found in protein.

Most viral inclusions which have been studied histochemically have exhibited positive reactions to stains for nucleic acids.[12,13] The absence of nucleic acid in ACN inclusions as demonstrated in this study greatly lessens the possibility that viruses play a role in the formation of the inclusions.

The protein nature of the ACN inclusions was directly demonstrated by the DNFB stain, which stains tyrosyl, amino and sulfhydryl groups, but not nucleic acids.[2] The specific stains for tyrosyl, amino, and sulfhydryl groups using the DNFB method gave a well defined positive reaction for tyrosine and sulfhydryl, but a weak reaction for amino groups. This was only a qualitative demonstration of these groups, and did not necessarily reflect their relative abundance. The existence of sulfhydryl groups in the inclusions was substantiated by the positive reaction to the DDD stain; this is reported to be a specific reaction.[10]

The ACN inclusions did not stain with the DDD or DNFB disulfide procedures, except for occasional weak positive reactions following DNFB. Because of the specificity of the DDD method, it is probable that the inclusions contain no disulfides or that the concentration of these is too low to be demonstrable. The occasional weak positive stain obtained with the DNFB procedure was probably due to incomplete

blockade of some amino or tyrosyl groups. On the basis of these results, it is not possible to eliminate disulfide linkages as constituents of the ACN inclusions with certainty. However, the presence of these linkages is consistent with the probable protein nature of the inclusions and with the existence of sulfhydryl groups.

The demonstration of tyrosine, sulfhydryl groups (cysteine), amino groups (lysine, glutamine or terminal amino), and possibly disulfide linkages (cystine) in the ACN inclusions indicated that protein or polypeptides were present. The physiologic mechanism responsible for the formation of these crystalline inclusions is unknown; it may involve altered permeability of the nuclear membrane or excess protein or polypeptide synthesis within the nucleus. It is possible that the ACN inclusions were formed post mortem by the aggregation of protein-like constituents of the nucleus in cells which had a high rate of protein synthesis just prior to death; but this is not likely in view of the extreme distention of the nuclear membrane in some instances, as well as the margination of chromatin. The histologic appearance is that of a process which occurred *in vivo*.

The conclusions which are drawn from this study are that the ACN inclusions contain reactive groups normally associated with the presence of protein or polypeptides. Other compounds normally occurring in the liver and kidney epithelial cells and capable of detection by the histochemical methods used in this and the previous study were not demonstrable.

Summary

Acidophilic, crystalline intranuclear inclusions observed within the epithelial cells of the liver and kidneys of 9 dogs were shown to contain reactive groups normally associated with the presence of proteins or polypeptides. Staining schedules are given for the demonstration of specific protein groups in formalin and alcohol fixed, paraffin-embedded tissue sections, utilizing a dinitrofluorobenzene technique.

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[*Illustrations follow*]

LEGENDS FOR FIGURES *

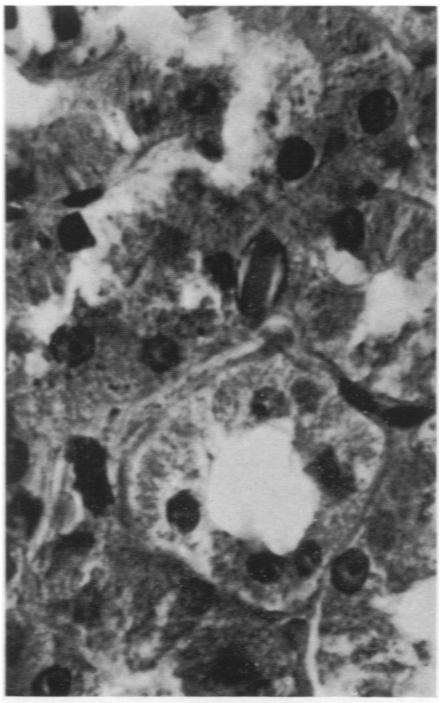
FIG. 1. Kidney of a dog. Single ACN inclusion in a renal tubular epithelial cell. Section stained with tetrazotized benzidine coupled with betanaphthol to demonstrate tyrosine, histidine, tryptophan and the purines and pyrimidines of nucleic acids. × 1000.

FIG. 2. Kidney of a dog. Two unstained ACN inclusions in renal tubular epithelial cells. Section treated by benzoyl chloride to block tyrosine, histidine and tryptophan and stained with tetrazotized benzidine coupled with betanaphthol. × 1000.

FIG. 3. Kidney of a dog. Single ACN inclusion in a renal tubular epithelial cell, stained with dinitrofluorobenzene coupled with H-acid to demonstrate tyrosyl, amino and sulfhydryl groups. × 1000.

FIG. 4. Liver of a dog. Single ACN inclusion in hepatic epithelial cell. Section treated by hydrogen peroxide to block sulfhydryl groups and nitrous acid to block amino groups. The dinitrofluorobenzene stain, coupled with H-acid was employed to demonstrate tyrosyl groups. × 1000.

* United States Army photographs, taken by Mr. William Hummel of the Pathology Service, Fitzsimons Army Hospital, Denver, Colo.



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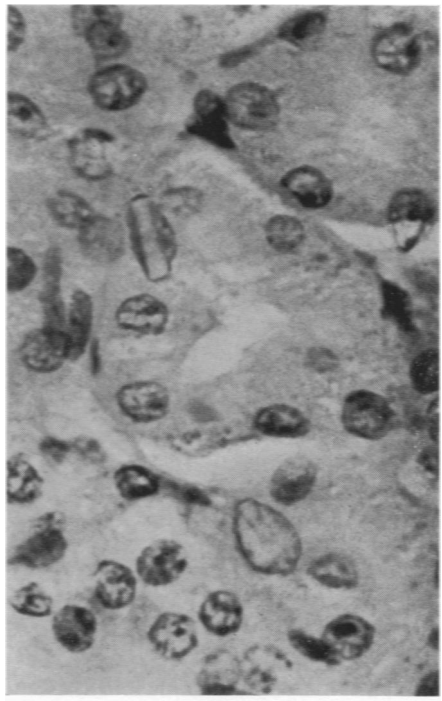

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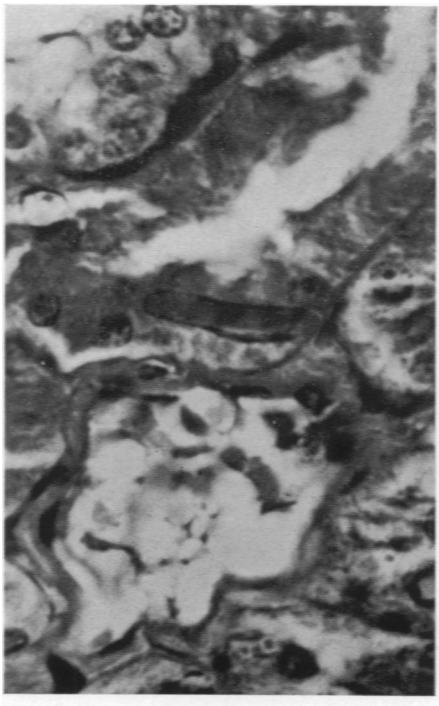

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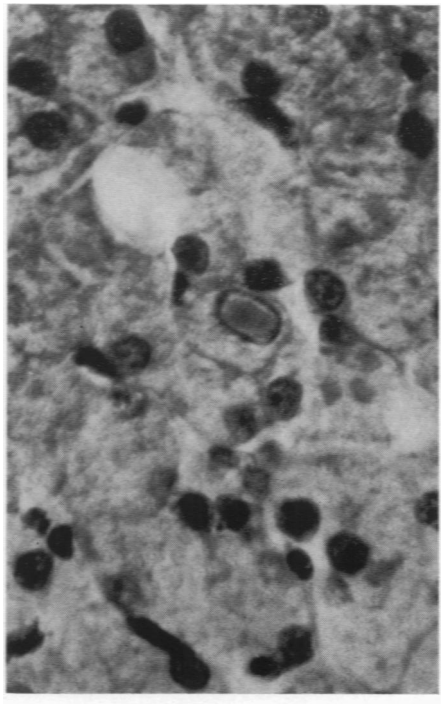

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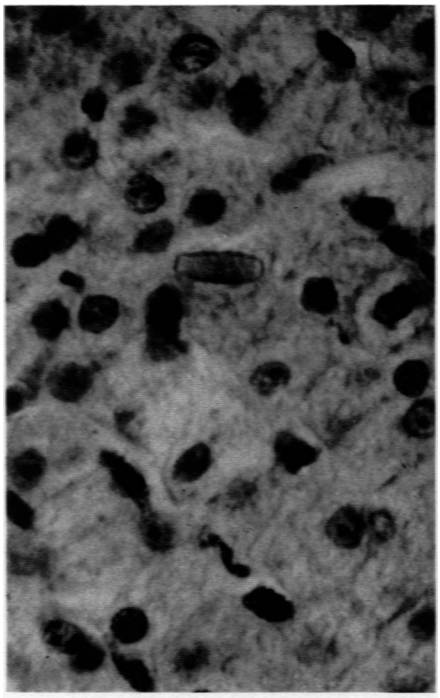
FIG. 5. Liver of a dog. Single ACN inclusion in a hepatic epithelial cell. Section treated by hydrogen peroxide to block sulfhydryl groups and diazoaniline to block tyrosyl groups. The dinitrofluorobenzene stain coupled with H-acid was employed to demonstrate the presence of amino groups. × 1000.

FIGS. 6 and 7. Liver and kidney of a dog. Single ACN inclusions are shown in epithelial cells of each organ. Sections treated by diazoaniline to block tyrosyl groups and nitrous acid to block amino groups. The dinitrofluorobenzene stain coupled with H-acid was employed to demonstrate the presence of sulfhydryl groups. × 1000.

FIG. 8. Kidney of dog. Single ACN inclusion in a renal tubular epithelial cell. The presence of sulfhydryl groups is demonstrated by the 2, 2′-dehydroxy-6, 6′ dinaphthyl disulfide stain. × 1000.



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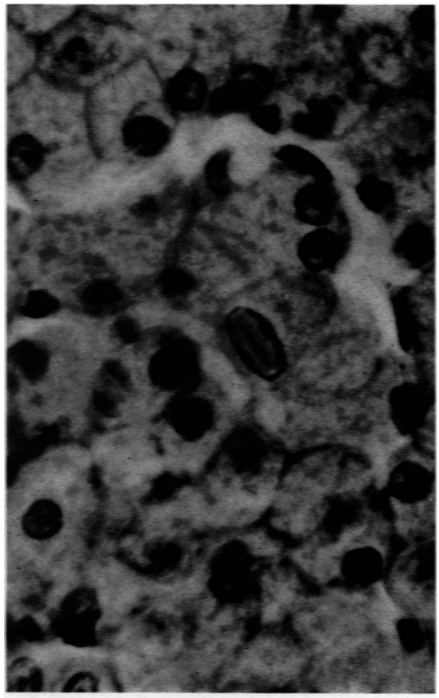

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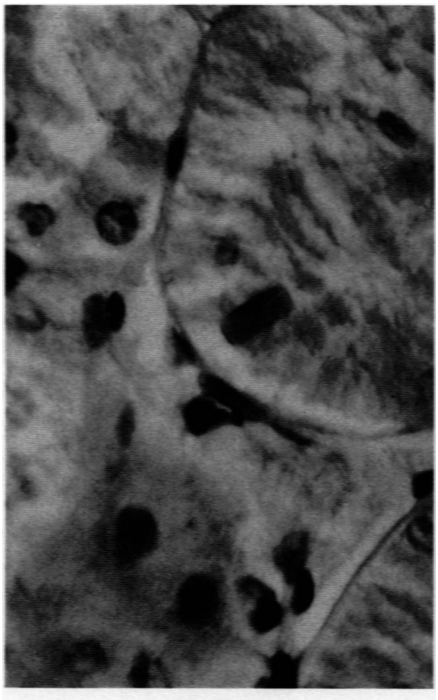

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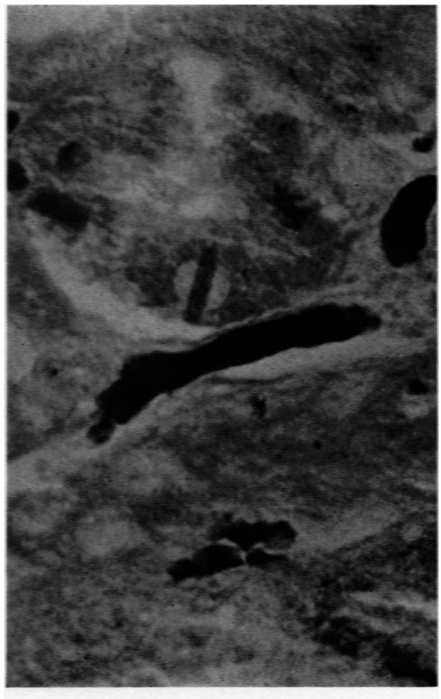

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