PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 165, 241–247 (1980)

The Histochemical Distribution of Alcohol Dehydrogenase at Selected Sites in Four Species of Experimental Animal (40964)

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Abstract. In view of the ascribed involvement of alcohol dehydrogenase (ADH) in the metabolic activation of the carcinogen methylazoxymethanol, the distribution of this enzyme in the ear canal, duodenum, ileum, and colon of rats, mice, hamsters, and guinea pigs was examined by a histochemical method using 1-butanol as substrate. The technique described gave reproducible and specific results. While the distribution and intensity of staining for ADH differed from organ to organ, corresponding organs from each of the four species examined showed a remarkable degree of similarity in the distribution and intensity of staining. However, major differences are known to exist in the sensitivities of these organs in the four species to the carcinogenicity of methylazoxymethanol and/or its metabolic precursors 1,2-dimethylhydrazine and azoxymethane. Thus, we conclude that no specific correlation exists between the overall distribution of ADH, as determined by our histochemical method, and the organ specificity of these carcinogens.

It was suggested by Schoental (1) that the enzyme alcohol dehydrogenase (ADH) may play a significant role in the metabolism of carcinogens such as methylazoxymethanol (MAM), a metabolite of the colon carcinogens 1,2-dimethylhydrazine (DMH), azoxymethane, and methylazoxymethanol acetate. This hypothesis received support from the work of Grab and Zedeck (2) who using both ethanol and MAM as substrate showed that NAD-dependent ADH activity was highest in those segments of rat intestine which were also most sensitive to the carcinogenic effects of methyl-azoxymethanol acetate. However, other studies have shown significant levels of ADH activity in many other rat tissues (3, 4). Also, Mistilis and Garske (5) demonstrated relatively high activity of this enzyme in liver, stomach, and small intestine but found no activity in the colon of Sprague–Dawley rats. Therefore, this study was undertaken to determine the anatomical distribution of ADH at selected sites in four species of experimental animal in which the response to the carcinogen DMH is known.

Materials and methods. Animals. Techniques were developed using tissues from adult male Fischer 344 rats (Charles River Breeding, Wilmington, Mass.). Final tissue evaluations were carried out on tissues of five adult male Fischer 344 rats (Charles River Breeding), five adult male Ha/ICR mice (ARS/Sprague–Dawley, Madison, Wisc.), five adult male Syrian golden hamsters (ARS/Sprague–Dawley) and three adult female Strain 2 guinea pigs (Frederick Laboratories, Fort Detrick, Md.).

Chemicals. Nitroblue tetrazolium (NBT) Grade III, β-nicotinamide adenine dinucleotide (NAD) Grade V, polyvinylpyrrolidone (PVP; PVP-10, average molecular weight 10,000), niacinamide, and iodoacetic acid were obtained from Sigma Chemical Company (St. Louis, Mo.), 1-butanol and magnesium chloride were obtained from Fisher Scientific Company (Pittsburgh, Pa.), and pyrazole was obtained from Eastman Kodak Company (Rochester, N.Y.).

Preparation of tissues for staining. Animals were killed by CO_2 overdose and portions of descending colon, ileum, duodenum, and inner ear canal were placed in Hanks' buffered saline solution at 4°C briefly until suitably sized portions, approximately 1 × 0.3 cm, could be cut and rapidly frozen in a drop of water containing 10 mg/ml PVP on a cryostat block precooled to −20°C. Multiple sections of each tissue were cut at 8–10 μm in a Pearse Slee G2-MR cold microscope cryostat and attached to clean glass microscope slides by momentary thawing.

0037-9727/80/100241-07$01.00/0
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Staining: 1. ADH. The method finally adopted was a modification of those used by Pearse (6) and Ferguson (3). Staining was carried out for 1 hr in a Coplin jar agitated in a water bath maintained at 37°C. The staining solution contained phosphate buffer, 15 m*M*, pH 7.4, NBT, 1.2 m*M*, $MgCl_2$, 5 m*M*, niacinamide, 1 m*M*, NAD, 3 m*M*, and 1-butanol, 0.386 *M*. After incubation in the staining mixture, slides were washed in physiological saline for 1 min, fixed in 10% buffered formalin acetate (pH 7.0–7.1) for 10 min, lightly counterstained in 0.25% aqueous safranin O (Fisher Scientific Co., Fair Lawn, N.J.) for 30 sec, washed in physiological saline for 1 min, rapidly dehydrated through graded alcohols to xylene, and then coverslipped using Coverbond (Scientific Products, McGaw Park, Ill).

2. ADH with inhibitor. Staining was carried out as for ADH but with the addition of 0.386 *M* pyrazole to the staining solution.

3. No-substrate control. Staining was carried out as for ADH but without 1-butanol in the staining mixture.

4. *Hematoxylin and eosin.* After fixation in 10% buffered formalin acetate (pH 7.0–7.1), slides were stained with hematoxylin and eosin by routine methods.

5. Miscellaneous procedures. Using sections of rat tissues including kidney, liver, colon, and ileum, we determined the effects of: (i) increasing buffer molarity to 0.05 *M*, and increasing the pH to 8.0; (ii) substituting Tris buffer 0.05 *M* (pH 7.6) in place of the phosphate buffer; (iii) omitting NAD from the stain; (iv) shortening the incubation time to 20, 10, and 5 min; (v) incubation at room temperature; (vi) omitting the counterstain; (vii) omitting the dehydration of sections following staining and coverslipping with a water-soluble medium; (viii) adding 0.01 *M* sodium cyanide to the staining solution (7); (ix) substituting ethanol 1.0 *M* and 0.1 *M* for 1-butanol; (x) adding PVP (75 mg/ml) to the staining solution; (xi) adding 0.1 *M* iodoacetic acid to the staining solution; and (xii) freezing tissues in liquid nitrogen followed by storage for 1–14 days at −80°C. In addition, to examine for the possibility of incomplete stain penetration, sections of rat colon and ileum were cut from tissue mounted flat with the mucosal surface up on cryostat blocks. Smears were made of scrapings taken by a glass microscope slide drawn across the mucosal surface of the bowel. These smears and sections of the bowel after scraping were also stained for ADH activity.

Results. Hematoxylin and eosin sections confirmed the normal appearances of the tissues under study and also confirmed the adequacy and orientation of the sections.

A summary of the results for ADH staining is given in Table I. The intensity of staining was graded visually on an arbitrary scale of 0 to +++. The grade of 0 was given where no staining was observed and ± was given where there were only occasional blue cytoplasmic granules present in some cells. An example of intense (+++) staining is shown in Fig. 1. In all tissues the staining appeared to be throughout the cytoplasm and was granular in nature. Glands lining the ear canal showed heavy staining for ADH (Fig. 1), the epithelial cells lining the villi of the duodenum showed moderate staining (Fig. 2), and the epithelial cells lining the villi of the ileum and lining the surface of the colon (Fig. 3) showed weak staining. The cytoplasm of both goblet and border cells appeared to show staining. The epithelial cells lining the crypts in the duodenum (Fig. 2), ileum, and colon (Fig. 3) showed a gradation of staining with minimal or no activity at the base of the crypts and increasing activity toward the surface.

In the presence of pyrazole, a competitive inhibitor of ADH (8, 9), the intensity of staining appeared to be reduced by approximately one grade but was not completely abolished. In the tissues studied no staining was observed in the absence of substrate (Fig. 1) except at the site of folds in the section, and occasionally in keratin lining the ear canal and in cartilage adjacent to the ear canal.

No apparent effect on alcohol dehydrogenase staining was noted by increasing the buffer molarity, increasing the buffer pH, substituting Tris buffer for phosphate buffer, omitting the dehydration of sections, omitting the counterstaining, adding sodium cyanide to the staining solution, or adding PVP to the staining solution.

TABLE I. THE HISTOCHEMICAL DISTRIBUTION AND INTENSITY OF STAINING FOR ALCOHOL DEHYDROGENASE IN TISSUES FROM FOUR SPECIES OF EXPERIMENTAL ANIMAL

	Rats F344	Mice Ha/ICR	Syrian golden hamster	Strain 2 guinea pigs
Ear canal				
Glandular epithelial cells	++−+++[a]	++−+++	++−+++	+++
Duct epithelium	0	0	0−±	0−±
Epithelium lining ear canal	0−±	0−±	0	0−+
Duodenum				
Villous epithelium	++	++	+−++	++
Crypt epithelium	0−±	0−±	0−+	0−±
Ileum				
Villous epithelium	±−+	±−+	+	+−++
Crypt epithelium	0−±	0−±	0−±	0−±
Descending colon				
Surface epithelium	±−+	±−+	+−++	+−++
Crypt epithelium	0−±	0−±	0−±	0−±

[a] The intensity of staining was graded visually on a scale of 0, ±, +, ++, and +++. 0 = no staining; ± = minimal and irregular staining; + = weak staining; ++ = moderate staining; +++ = heavy staining.

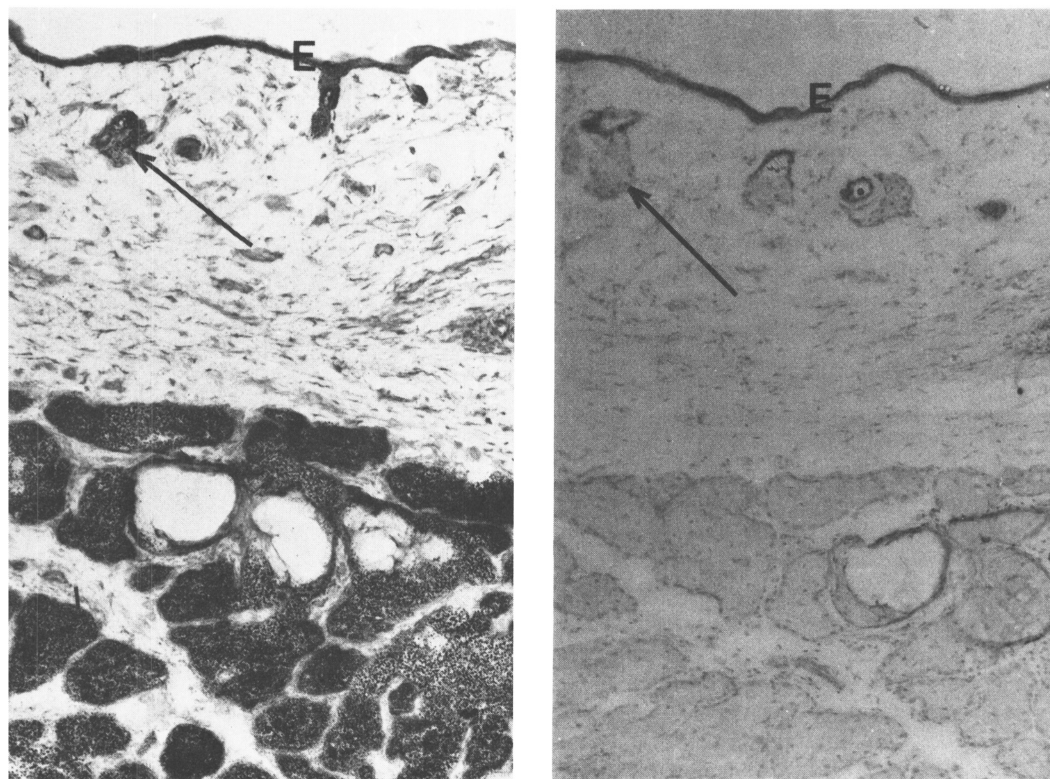

FIG. 1. Photomicrographs of cryostat sections of the inner, posterior wall of the ear canal of an F-344 rat. The sebaceous gland (of Zymbal) occupies the lower half of each field and smaller sebaceous glands of the posterior wall are also present (arrows). The lumen of the ear canal is above and is lined by a keratinizing squamous epithelium (E). (Left) Section stained for ADH activity using 1-butanol as substrate. There is positive intense dark (blue) granular staining of cells of the sebaceous glands. ×310. (Right) Section stained for ADH activity but with substrate omitted from the incubation. Dark staining is absent and the tissue is outlined by the pale (red) counterstain. ×310.

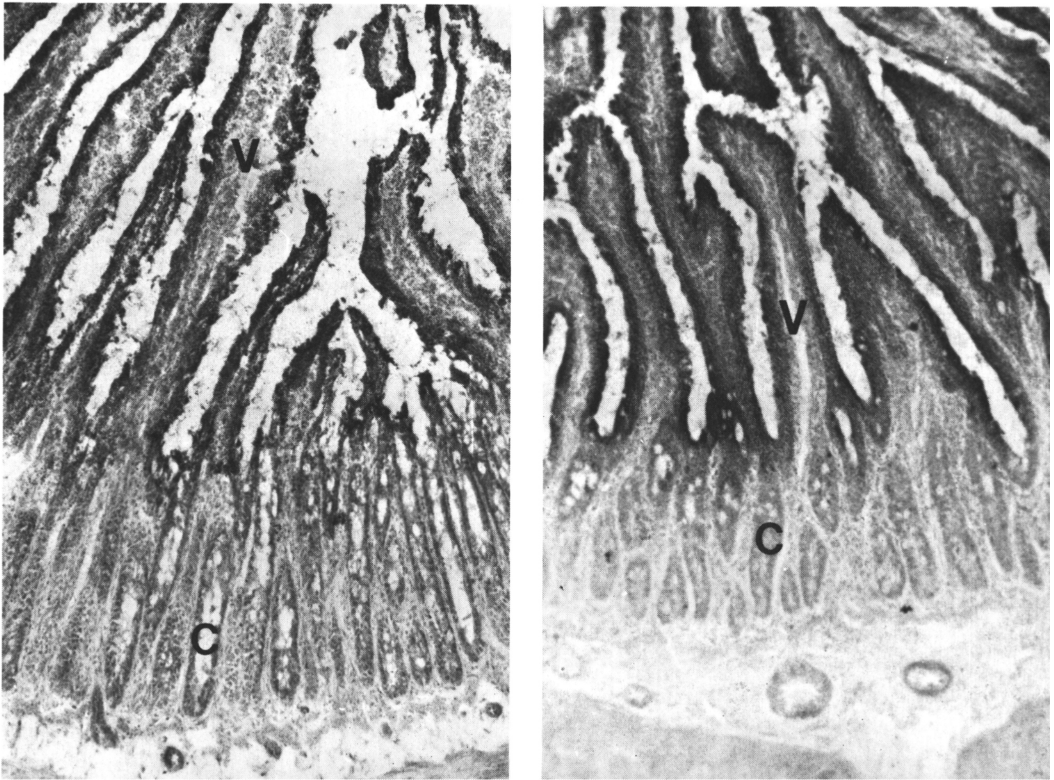

FIG. 2. Photomicrographs of cryostat sections of duodenum stained for ADH activity using 1-butanol as substrate. Cells covering the villi (V) show positive moderately intense dark (blue) granular staining but cells lining the crypts (C) lack positive staining. The distribution and intensity of staining is similar in both sections. (Left) From an F-344 rat. ×330. (Right) From a Strain 2 guinea pig. ×330.

Reduced or irregular staining was noted when the incubation period was reduced, when the incubation was carried out at room temperature, when ethanol rather than 1-butanol was used, and when the tissue had been stored at −80°C. However, staining was not observed in the absence of NAD or in the presence of the relatively nonspecific enzyme inhibitor iodoacetic acid (9).

Tangential sections and sections of previously scraped ileum and colon containing the lower parts of crypts showed a similar distribution and intensity of staining as observed in cross sections. Staining of smears made from the scrapings of the colon contained scattered whole isolated crypts and these also showed a gradient in staining from the top to the base of the crypts (Fig. 4).

Discussion. A satisfactory histochemical technique for the demonstration of ADH activity in intestinal and ear canal tissues is described and has been carefully evaluated in terms of the variables known to affect such enzyme histochemical reactions. In particular, in the tissues studied there was little or no staining in the absence of substrate, thus avoiding the complication of the so-called "nothing dehydrogenase" (10, 11) effect. Since this type of artifactual staining is more prominent at slightly alkaline pHs (10), we carried out the staining at pH 7.4 or 8 even though these values are below the pH optimum for the enzyme. Using electrophoretic methods, Koen and Shaw (12, 13) have shown that the "nothing dehydrogenase" activity was principally associated with ADH itself suggesting that this effect could be due to undetected substrates pres-

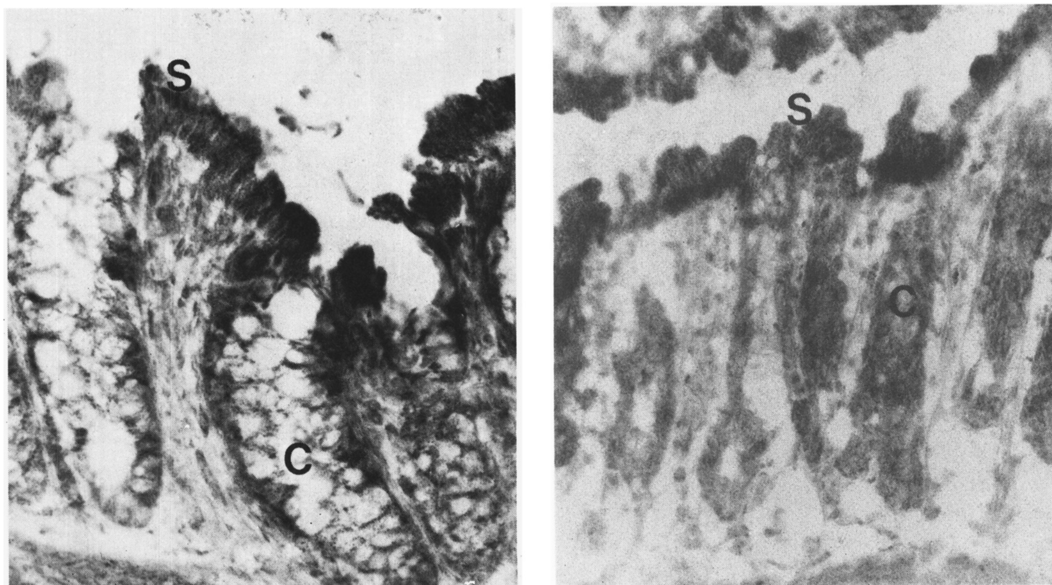

FIG. 3. Photomicrographs of cryostat sections of descending colon stained for ADH activity using 1-butanol as substrate. Cells on the surface (S) and cells lining the upper part of the crypts (C) show slightly variable positive dark (blue) staining of a fairly weak intensity. Positive staining of the cells in the bottom of the crypts is minimal. The distribution and intensity of staining is similar in both sections. (Left) From an F-344 rat. ×660. (Right) From a Strain 2 guinea pig. ×620.

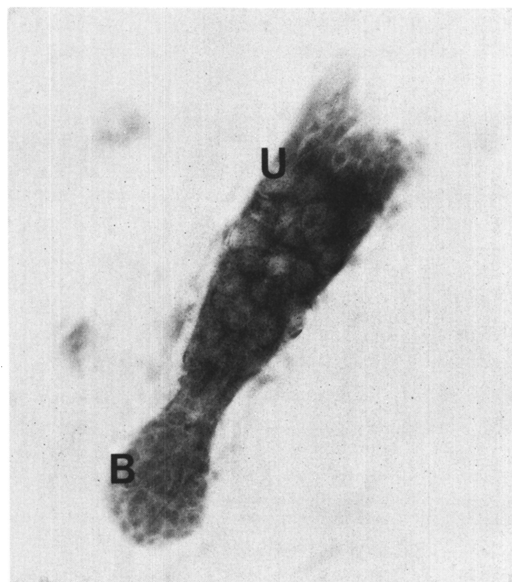

FIG. 4. Photomicrograph of a smear made of scrapings from the descending colon of an F-344 rat, and stained for ADH activity. An isolated colon crypt is present and shows positive dark (blue) granular staining of weak intensity in the cells in the upper part (U) of the crypt. Staining is minimal near the base (B) of the crypt. ×660.

ent either in the tissue or in the histochemicals used. It is, therefore, relevant to note that commercially available NBT may contain up to 15% ethanol (13) and that commonly used mounting media for cryostat tissue blocks contain glycols which are known to act as substrates for ADH (3).

Although the addition of PVP and cyanide to the incubating mixture and the avoidance of dehydration and of mounting coverslips with a non-water soluble mounting medium has been recommended (7), these modifications did not result in any detectable differences in staining in the present study.

Our observation of ADH activity in all of the epithelial tissues examined is in agreement with the reported observations of a widespread distribution of ADH in organs other than the liver, determined both histochemically (3, 4, 15) and biochemically (16–18). While some of these reports have described the presence of ADH activity in the intestine (3, 16–18), none has systematically examined the histochemical distribution of this enzyme in the various an-

atomical subdivisions of the organ. Also, to our knowledge, the presence of ADH in the glands of the ear canal has not been previously reported.

In all the tissue examined, positive granular staining appeared to be widespread within the cytoplasm of those cells showing activity. Although the granularity of staining may suggest activity within cellular organelles, Pearse (7) has pointed out that granularity may be produced or enhanced by the type of processing of sections after staining has been completed. We, therefore, do not infer any particular subcellular distribution from our results except that activity did not appear to be present in nuclei.

In intestinal mucosa we observed maximum ADH activity in cells on the villi in the small bowel and in cells on the surface and in the upper one-third of the crypts of Lieberkuhn in the large bowel. This preferential distribution in the more mature cells of the mucosa parallels the distribution reported for several other mucosal enzymes including γ-glutamyltransferase (19), adenosine deaminase, adenine and hypoxanthine phosphoribosyltransferases, purine nucleoside phosphorylase, and thymidylate phosphatase (20). The possibility of an artifactual distribution of staining caused by inadequate stain penetration was considered because of the predominantly surface localization of staining in intestinal tissues but was excluded by the demonstration of the same differential staining in semitangential sections of the mucosa and in isolated crypts. It is of interest to note that while DMH is thought to act on the actively dividing cells in the lower parts of the intestinal crypts (21), these cells contained very little ADH activity compared to cells in the upper portions of the crypts.

The pattern and intensity of staining in a given type of tissue was similar regardless of the species of origin. The maximum intensity of staining was observed in the glandular cells of the ear canal and only relatively weak staining was observed in the ileum and colon in all species examined. However, the incidence of tumors induced by DMH and its metabolites in these species varies considerably from tissue to tissue and species to species. In particular, tumors of the ear canal glands are induced with high incidence in rats with these carcinogens (22–24) but have not been observed in the other species. Similarly, tumors of the colon are induced frequently in rats (22, 23), mice (25–27), and hamsters (28–30) with these chemicals but not in guinea pigs (30, 31). Tumors are also induced by these carcinogens frequently in the duodenum but only occasionally in the ileum of rats (22, 23) and occasionally in the duodenum and ileum of hamsters (28), but not in the duodenum or ileum of mice (25–27) and guinea pigs (30, 31).

The present study indicates that no specific correlation exists between the presence in an organ of ADH as determined histochemically using 1-butanol as substrate and the susceptibility of that organ to the carcinogenicity of DMH or its metabolites, azoxymethane and methylazoxymethanol. However, in view of the increasing evidence that ADH does play a role in the metabolism of methylazoxymethanol (2, 32), there is still the possibility that quantitative or qualitative differences not detectable by histochemical techniques may play a role in determining the organ specificity of these carcinogens. Such differences, for example, could be associated with the presence of a tissue-specific ADH isoenzyme with a high affinity for methylazoxymethanol. These possibilities need to be considered and are currently under investigation in this laboratory.

The authors wish to acknowledge the helpful advice of Dr. G. D. McCoy, the provision of facilities by Dr. G. M. Williams, the assistance of Mr. C.-Q. Wong with preparation of the photomicrographs, and the assistance of Mrs. E. M. Harvey with the preparation of the manuscript. This work was supported in part by Contract NO1 CP 75948 from the National Cancer Institute and in part by Public Health Service International Research Fellowship 1 FO5 TW 2666-01.

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Received April 3, 1980. P.S.E.B.M. 1980, Vol. 165.