IN VITRO
Volume 14, No. 10, 1978

EXPLANT CULTURE OF RAT COLON: A MODEL SYSTEM FOR STUDYING METABOLISM OF CHEMICAL CARCINOGENS

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SUMMARY

An explant culture system has been developed for the long-term maintenance of colonic tissue from the rat. Explants of 1 cm^2 in size were placed in tissue-culture dishes to which was added 2 ml of CMRL-1066 medium supplemented with glucose, hydrocortisone, β-retinyl acetate, and either 2.5% bovine albumin or 5% fetal bovine serum. The dishes were placed in a controlled-atmosphere chamber which was gassed with 95% O_2 and 5% CO_2. The chamber then was placed on a rocker platform which rocked at 10 cycles per min causing the medium to flow intermittently over the epithelial surface. The explants were incubated at 30° C. The viability of the tissue was measured both by incorporation of specific precursors into cellular macromolecules and by monitoring of tissue morphology with light and electron microscopy. Cultured rat colon was able to metabolize benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, aflatoxin B_1, dimethylnitrosamine, 1,2-dimethylhydrazine, and methylazoxymethanol acetate into chemical species that bind to cellular DNA and protein.

Key words: colon; explant culture; carcinogen metabolism.

INTRODUCTION

Cancer of the colon is one of the most common cancers in the industrialized world. Epidemiological studies indicate that colonic cancer is related mainly to dietary factors including fat and micronutrients (1-3). An animal model system for investigating these factors has been developed (4,5). Intrarectal administration of either N-methyl N′-nitro-N-nitrosguanidine or 1,2-dimethylhydrazine to rats caused adenocarcinoma which was histologically quite similar to colonic adenocarcinoma in man (4-6). Similar results were found when azoxymethane was given by subcutaneous administration (5).

The extrapolation of carcinogenesis data from studies utilizing experimental animals to man presents a complex problem. A new approach to provide a link between the experimental animal studies and human is to use in vitro systems that permit comparative studies under similar conditions of exposure to carcinogens, e.g. explant cultures of human and rat colon. In our laboratories explant systems have been developed for culturing human target tissues; i.e. bronchus (7), pancreatic duct (8) and colon (9), and explants of human colon were able to metabolize procarcinogens of several chemical classes, e.g. benzo[*a*]pyrene (BP), dimethylnitrosamine (DMN), and 1,2-dimethylhydrazine (1,2-DMH) (10).

In the present report, the development of an explant system for rat colonic tissue is described. Three major variables that permitted the long-term culturing of rat colonic tissue were (a) increased O_2 tension; (b) supplementation of the medium with glucose; and (c) lowering the incubation temperature to 30° C. The metabolism of various procarcinogens by rat colonic tissue cultured for 4 days was investigated. Recently, methods also have been described for the explant culture of colon from newborn rats, newborn hamsters (11), and adult mice (12).

MATERIALS AND METHODS

Animals. Male Charles River CD rats (Charles River, Wilmington, Mass.), 4- to 5-week-old,

were used for biochemical experiments; and adult male Fisher F-344 (300- to 350-g) also were used in the development of the methodology for explant culture. The animals were kept in cages with pine-wood sawdust as bedding in well ventilated rooms at 20° C with 12-hr dark/light cycles (7 a.m. to 7 p.m.). They were fed on Wayne Lab-blox (Allied Mills Inc., Chicago, Ill.) until fasted 1 day prior to killing.

Preparation of explants. The rats were killed by asphyxiation, and the descending colon from the splenic flexure to 1½ inch below the splenic flexure was removed by aseptic technique. The colon was washed three times with ice-cold L-15 medium containing penicillin G (100 U per ml), streptomycin (100 μg per ml), and amphotericin B (0.25 μg per ml), and immersed in L-15 medium at 4° C until cultured (tissue-culture medium and antibiotics were obtained from GIBCO, Grand Island, N.Y.). The colon was opened longitudinally with surgical scissors and cut into two explants: 7.5- by 7.5-mm for biochemical experiments, and 2.5- by 2.5-mm for long-term culture.

Culture conditions. Individual explants were placed in 60-mm plastic tissue-culture dishes (Falcon Plastics, Oxnard, Calif.) with the epithelium facing the gas-medium interface. The tissue was placed near the edge of the dish on an area that had been scratched with a sterile needle. Two ml of culture medium was added to each dish. The medium was CMRL-1066 supplemented with glucose (5 mg per ml; Sigma Chemical Co., St. Louis, Mo.); tricine buffer (pH 7.4; 10 mM; GIBCO); glutamine (3 mM); hydrocortisone hemisuccinate (0.5 μg per ml; Upjohn Co., Kalamazoo, Mich.); β-retinyl acetate (0.1 μg per ml; Hoffman-LaRoche, Nutley, N.J.); 5% fetal bovine serum (GIBCO), or 2.5% bovine albumin (Miles Laboratories Inc., Kankakee, Ill.); penicillin G (100 U per ml); streptomycin (100 μg per ml); gentamicin (50 μg per ml; Schering Corp., Kenilworth, N.J.); and amphotericin B (0.25 μg per ml). Amphotericin B was included only in the first two medium changes. The explants were incubated in an atmosphere of 95% O_2 and 5% CO_2 at 30° C on a rocker platform (10 cycles per min) so that the explant was submerged one-half of the time. The medium was changed at day 1 and then every 2 days.

Biochemical studies. [^{3}H]Thymidine (50 μCi per 2 ml; 21 Ci per mmol; Amersham/Searle, Arlington Heights, Ill.) or [^{3}H]leucine (50 μCi per 2 ml; 5 Ci per mmol; Amersham/Searle) was added to the cultures at the given time period and incubated for 4 hr. Two explants were fixed for autoradiography (ARG) and two were used for biochemical determinations. For the biochemical studies, the mucosal layers were scraped from the stroma, suspended and homogenized in 1 ml Tris-buffer for [^{3}H]leucine incorporation and in 1 ml 1 *N* NaOH for DNA determinations. Protein and DNA then were precipitated by addition of 1 *N* perchloric acid, and the pellets washed with 0.2 *N* perchloric acid until the radioactivity in the supernate was negligible. DNA concentration was determined by diphenylamine reaction (13) and protein by the Hartree method (14). Radioactivity was measured in a Beckman LS-335 scintillation counter using Aquasol (New England Nuclear Corp., Boston, Mass.) as the cocktail.

Activation of chemical carcinogens. After 4 days in culture one of the following labeled carcinogens was added to the culture medium: [^{3}H](G)BP (1.5 μM; 32 μCi per ml; 20 Ci per mmol; Amersham/Searle); [^{3}H]7,12-dimethylbenz[*a*]-anthracene (3.2 μM; 40 μCi per ml; 14.5 Ci per mmol; Amersham/Searle); [^{3}H]aflatoxin B_1 (0.5 μM; 6 μCi per ml; 13 Ci per mmol; Moravek Biochemicals, City of Industry, Calif.) dissolved in DMSO (final concentration 0.5%); [^{14}C]1,2-DMH (1.3 mM; 5 μCi per ml; 3.78 mCi per mmol; New England Nuclear Corp.); [^{14}C]methylazoxymethanol acetate (MAM; 0.26 mM; 10 μCi per ml; 39.5 mCi per mmol; J. Kepler, Research Triangle Institute) dissolved in L-15 medium; and [^{14}C]DMN (135 μM; 5 μCi per ml; 35 mCi per mmol; J. Kepler, Research Triangle Institute; prepared under NCI Contract N01-CP-55677); and purified by Dowex-1 sulfite chromatography according to the method of den Engelse, Gebbink and Philippus (15) and dissolved in water. After incubation of the explants with the carcinogen for 24 hr, except for MAM which was incubated only for 8 hr, the colonic mucosa was scraped from the supporting structure. Scrapings from five explants were pooled for each experimental variable. DNA was isolated by phenol extraction and purified on a CsCl gradient as previously described (16).

Electron microscopy and autoradiography. One explant from each variable was fixed in 3% glutaraldehyde both for monitoring cell viability by high-resolution light microscopy and for autoradiography (17). Colon explants were fixed in 3% glutaraldehyde buffered with 0.1 M s-collidine (pH 7.4) and postfixed in cold 1.33% OsO_4 buffered with 0.1 M s-collidine (pH 7.4) for 2 hr, dehydrated in ethanol and embedded in a mixture of

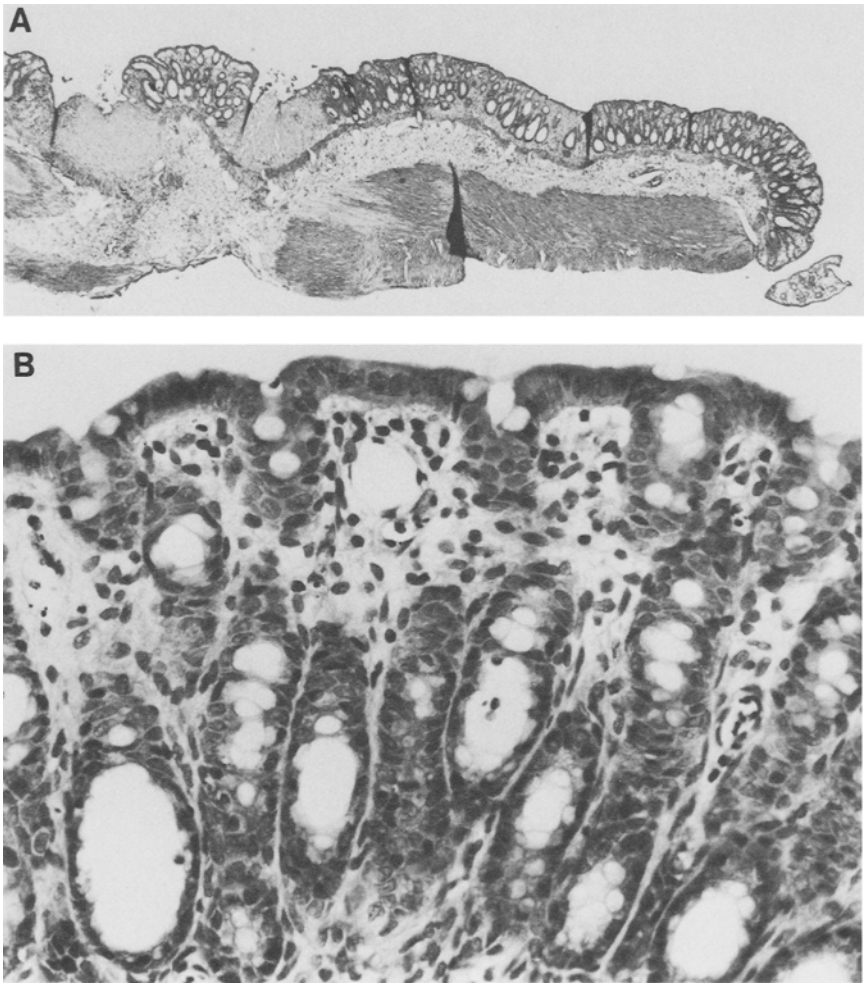

FIG. 1. Explant of rat colon cultured for 14 days. *A*, Well maintained glands and good integrity between mucosa, submucosa and muscular layer. H and E. ×27. *B*, The same tissue at higher magnification. A change from columnar to cuboidal epithelial cells is observed. Goblet cells are present in both the glands and at the luminal surface. H and E. ×350.

Epon-Araldite (15). Ultrathin sections were cut by an LKB microtome and double stained with lead acetate and uranyl nitrate. The sections were examined in a Hitachi Model 12 electron microscope at 75 kV. For autoradiography, 1-μm sections were placed on glass slides and coated with Eastman Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.) at 45° C, dried and stored in total darkness at 4° C with desiccant for 4 weeks. The slides were developed and fixed as previously described (18).

RESULTS

One of the initial problems in culturing rat colon was a rapid lowering of the pH of the medium, which was caused by production of lactic acid. This was resolved, in part, by increasing the O_2 tension from 20% to 95%. The addition of a tricine buffer (20 mM) also helped to stabilize the pH of the culture medium.

Several different media, i.e. Parsa, L-15, Ham's F-12 and CMRL-1066, were tested initially for their ability to support the growth of the explants. CMRL-1066 was chosen because it gave more consistent results in maintaining colonic explants for longer periods of time in culture.

The vitamin and hormone concentrations in the medium were similar to those used in our bronchial explant system (7). No improvement in either survival or ultrastructural features of the

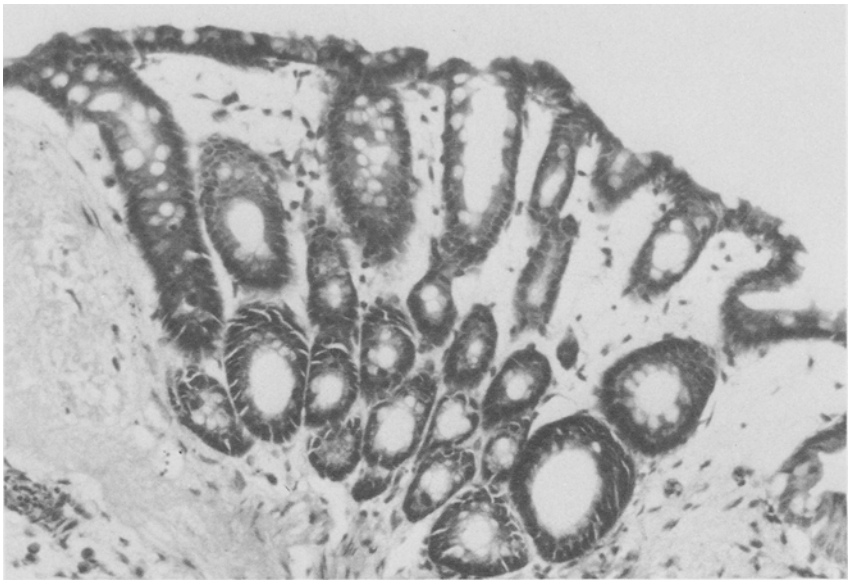

FIG. 2. Twenty-one days in culture. A progressive change towards cuboidal epithelial cells and fewer mucous cells is seen. H and E. ×220.

explants was observed by varying the concentrations of hydrocortisone (0.5 to 1.0 μg per ml), insulin (0 to 50 μg per ml), or β-retinyl acetate (1 to 10 μg per ml). Inclusion in the culture medium of thyroxine (0.1 to 1.0 μg per ml), which affects the differentiation of intestinal cells in animals (19), had no observable effect on the maintenance of rat colon.

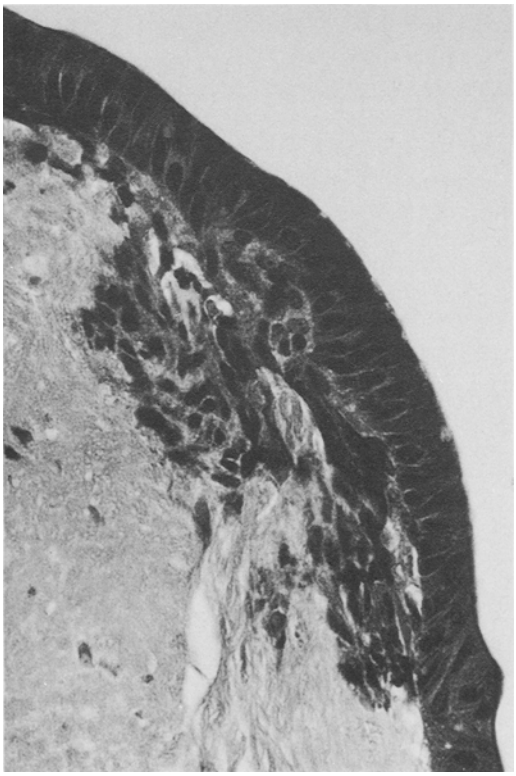

FIG. 3. Explant of rat colon cultured for 63 days. The glands have disappeared and only a single layer of epithelial cells are lining the surface. Cells containing mucus are seen. H and E. ×1190.

Several types of sera were examined for their ability to sustain explant survival, i.e. fetal bovine serum, horse serum and rat serum in concentrations ranging from 1% to 20%. The best results were obtained by using either fetal bovine serum (5%) or bovine albumin (2.5%). The latter was used for the metabolism experiments since it permitted the use of a more chemically defined system.

The normal appearance of the colonic epithelium was maintained for at least 63 days under these culture conditions. A difference in survival time among different anatomical segments was noted; the epithelium of the rectum and the descending colon was preserved for a longer period of time than the other segments. A colon explant cultured for 14 days is shown in Fig. 1; the explants showed well maintained intestinal glands and good integrity of the structural relationship between mucosa, submucosa and muscular layers. However, when compared to samples prior to culture, there was some decrease in the number of goblet cells (18) (Fig. 1*A*). A change from columnar to cuboidal epithelium also was observed (Fig. 2). Formation of intestinal glands was observed throughout the culture period; these

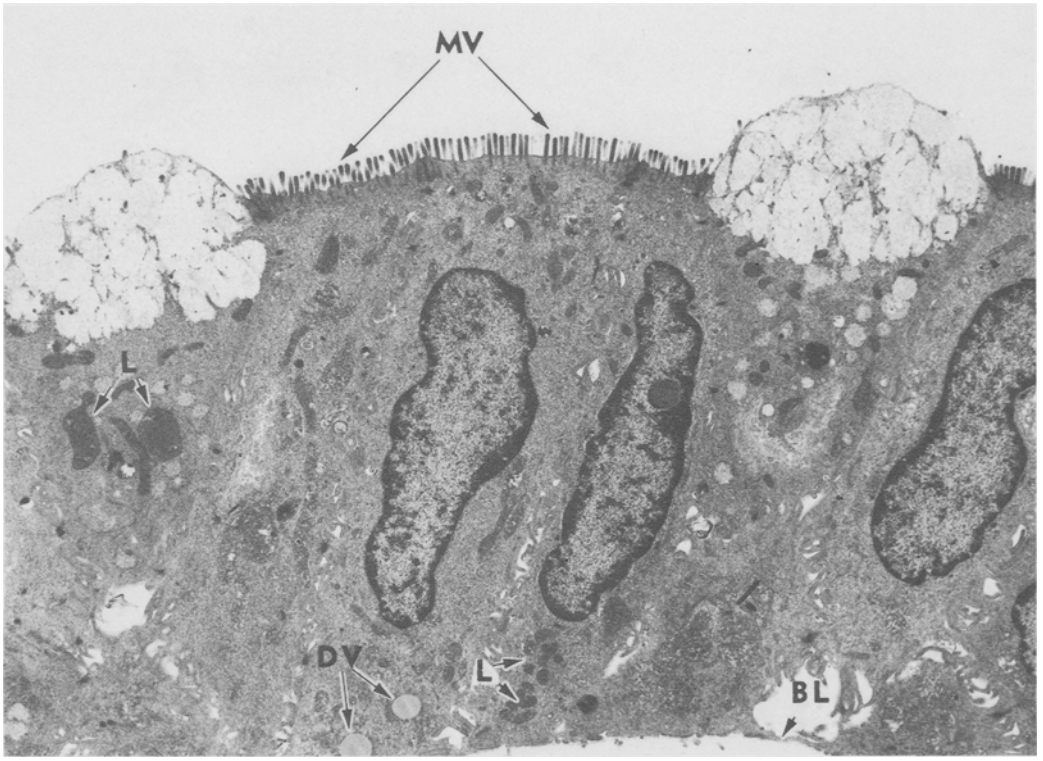

FIG. 4. Electron micrograph of explant of rat colon cultured for 14 days. The explant has both absorptive and mucous cells which have microvilli covered with well developed glycocalyx and several lysomes. ×3000.

glands became progressively shorter and wider and, after 31 days in culture, just a single layer of epithelial cells was observed (Fig. 3). Electron micrographs of rat colon explants cultured for 14 days show both columnar absorptive cells and mucus-secreting cells (Fig. 4). The absorptive cells had numerous microvilli covered with a well developed glycocalyx and contained several lysosomes and digestive vacuoles close to the basal lamina. After 5 weeks in culture, the explants still had well preserved microvilli and prominent Golgi apparatus; however, the mitochondria appeared mildly condensed (Fig. 5). Goblet cells were still observed at this time (Fig. 6). Incorporation of precursors into macromolecules was demonstrated up to 21 days (Table 1). Autoradiograms show that both [^{3}H]Tdr (Fig. 7) and [^{3}H]Leu (Fig. 8) were incorporated into epithelial cells lining the luminal surface and the crypts; the highest number of labeled nuclei are in the lower part of the crypt.

Cultured rat colon was able to metabolize several procarcinogens into chemical species which formed covalent bonds with cellular DNA (Tables 2, 3). Of the procarcinogens whose ultimate form is an epoxide, AFB_1 had a higher binding level than either BP or DMBA. The highest binding level was observed with 1,2-DMH. BP was bound uniformly to the intestinal epithelial cells (Fig. 9).

TABLE 1

INCORPORATION OF [^{3}H] LEU AND [^{3}H]TDR INTO CELLULAR PROTEIN AND DNA[a]

Day	[^{3}H]Leucine	[^{3}H]Thymidine
	dpm/μg protein	*dpm/μg* DNA
0	739	1105
1	988	672
2	1079	1218
3	559	1025
5	579	1206
7	482	1111
10	324	2266
14	855	1685
17	867	1041
21	636	988

[a] [^{3}H]Leu (50 μCi per ml) or [^{3}H]TdR (50 μCi per ml) was added to the cultures at the given time period and incubated for 4 hr. The mucosa was removed by scraping, and the protein and DNA were isolated from the acid-insoluble material (1 *N* perchloric acid).

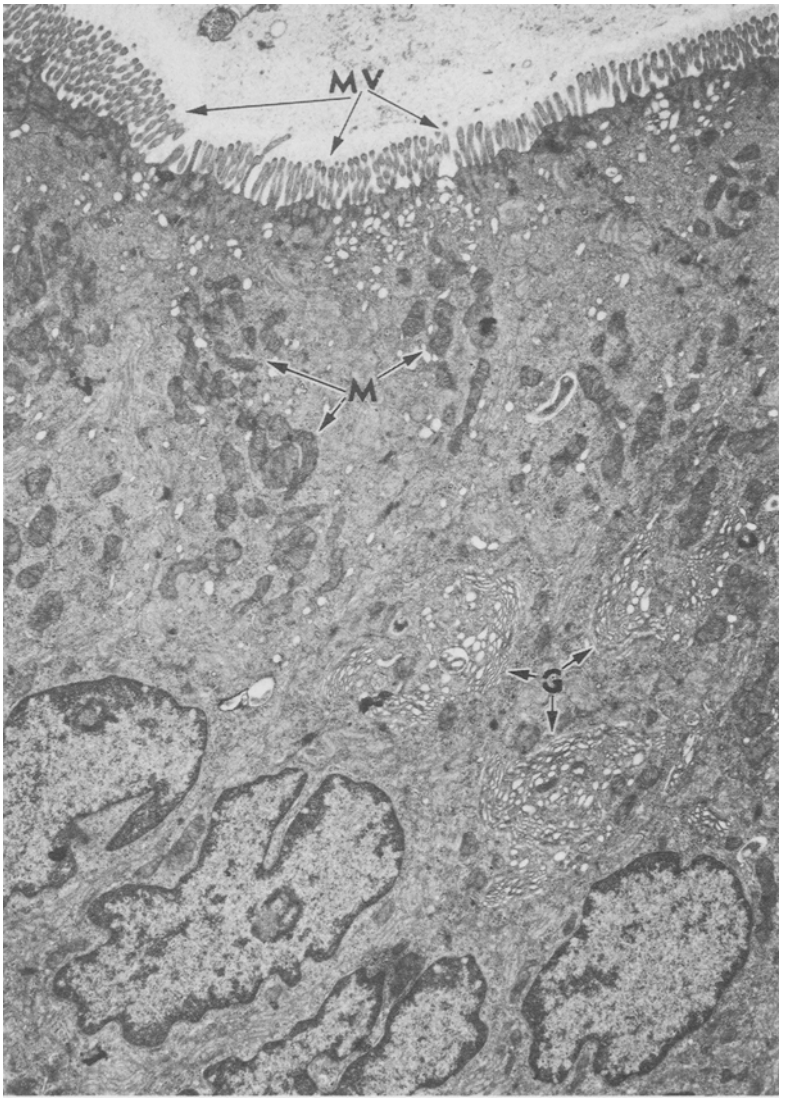

FIG. 5. Electron micrograph of explant of rat colon cultured for 35 days. The epithelial cells have microvilli and prominent Golgi complexes. Mitochondria appear mildly condensed. ×5000.

DISCUSSION

The development of an explant culture system for the long-term maintenance of rat colonic tissues has been described. The rat colon was capable of metabolizing various classes of procarcinogens to forms that were bound to cellular DNA and protein. Similarly, we have reported that human colon could be maintained in explant culture and was capable of activating various classes of procarcinogens; i.e. polynuclear aromatic hydrocarbons, N-nitrosamines and dimethylhydrazine (9, 10). Elevation of the atmospheric O_2 tension to 95% and supplementation of the medium with glucose were two factors that were important for the long-term maintenance of both rat and human colonic explants. However, colonic tissues from the two species differed with respect to the temperature of incubation; i.e. the survival of rat-colon explants was more optimal at 30° C than at 36.5° C and the opposite was found with human-colon explants. Although the reason(s) for this is unknown, it is possible that at 36.5° C, rat colon, as opposed to human colon, metabolizes substrates to lactic acid so rapidly that the accumulated lactic acid is highly toxic. Even at 30° C, it was necessary to buffer the culture medium for rat colonic explants to permit optimum

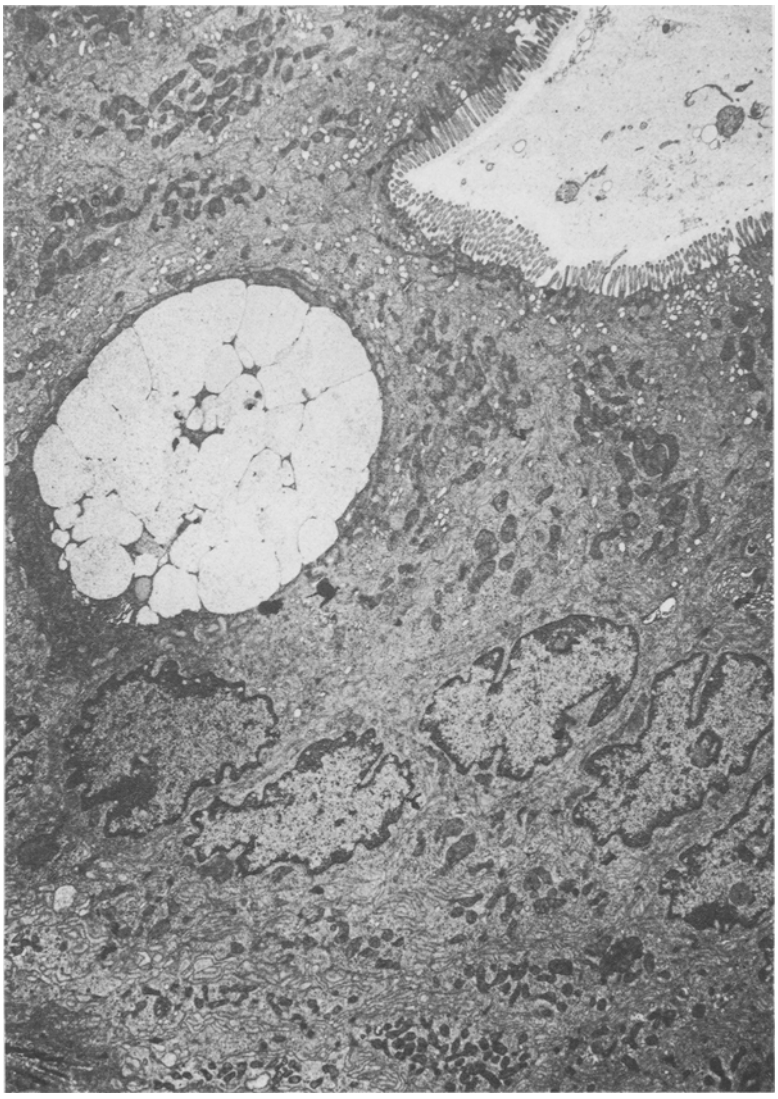

FIG. 6. Explant cultured for 35 days shows both absorptive and mucous cells. ×3000.

survival. The culture conditions described here were used successfully for both weanling and adult rats. In all experiments, explants from rectum and descending colon were maintained more optimally than those from ascending or transverse colon. Massive necrosis of the ascending and transverse colon was observed after a few days followed by repopulation of the epithelium to form a single layer of cells over the stromal matrix.

Incorporation of precursors into DNA and into protein was found throughout the culture period. ARG indicated that [^{3}H]Leu was incorporated in both epithelial cells on the luminal surface and in the crypts, the highest level of activity being in the lower part of the crypts. The highest number of labeled nuclei also were found in the lower part of the crypts, similar to results from in vivo studies in the rat (20).

A major advantage of our explant culture system for colon is that it permits the long-term maintenance of explants as large as 1 cm^2. It is likely that the intermittent exposure of the colonic explants to the atmosphere and the medium allows optimal diffusion of gases and nutrients to the tissue, thus permitting the maintenance of larger explants than are commonly used in explant

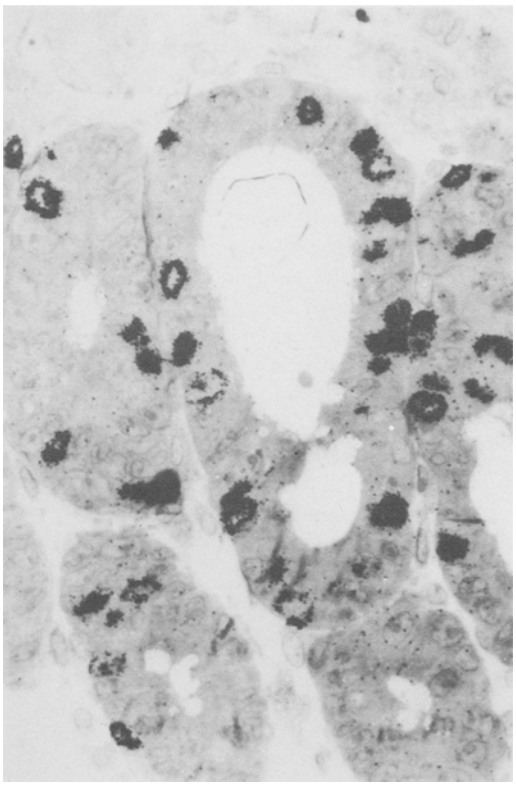

FIG. 7. Incorporation of [^{3}H]Tdr into rat-colon explant cultured for 14 days. Toluidine blue. ×540.

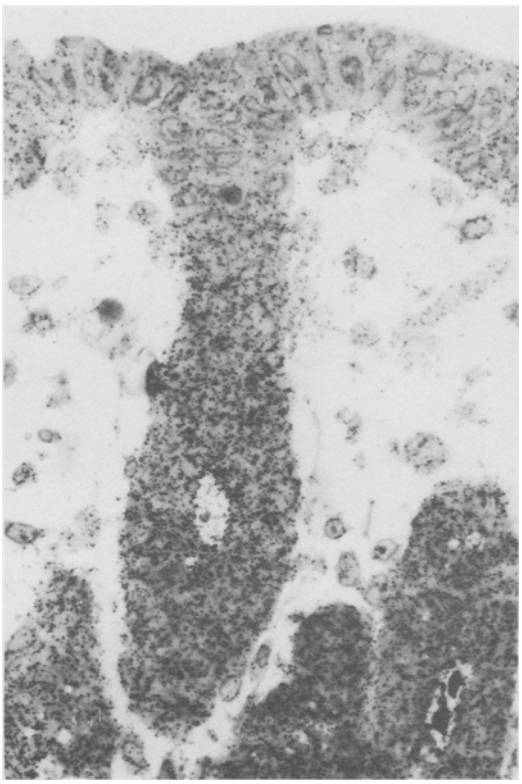

FIG. 8. Incorporation of [^{3}H]Leu into explant cultured for 14 days. Toluidine blue. ×540.

culture. The culture conditions described here were used successfully for both weanling and adult rats, and the explants were maintained for at least 63 days. In contrast, at the outset of these studies, we attempted to use the method of colon explant culture described by Schiff (11) and found it to be unsuitable for maintaining explants of 1 cm^2 for more than 12 to 24 hr. Biochemical investigations are greatly simplified through the use of explants of this size.

Several chemical carcinogens have been found to induce colonic cancer (3, 21). There is evidence that the procarcinogens can be enzymatically activated in (a) organs other than the colon and reach

TABLE 2

METABOLISM OF CHEMICAL CARCINOGENS BY CULTURED RAT COLON[a]

	pmol Carcinogen-Bound/10 mg DNA
Benzo[*a*]pyrene (1.5 μM)	3.5 ± 0.3[b]
7,12-Dimethylbenz[*a*]anthracene (3.2 μM)	5.2 ± 0.5[c]
Aflatoxin B_1 (0.5 μM)	125 ± 2[c]
Dimethylnitrosamine (0.135 mM)	845 ± 59[c]
1,2-Dimethylhydrazine (1.3 mM)	13,526 ± 323[b]

[a] Rat colon was cultured 4 days prior to addition of the carcinogens. After-incubation for 24 hr, the mucosa was removed by scraping, and the DNA was isolated by the phenol-extraction procedure and purified on a CsCl gradient.

[b] Mean ± S.E.M. of seven determinations.

[c] Mean ± S.E.M. of three determinations.

TABLE 3

METABOLISM OF [^{14}C]1,2-DIMETHYLHYDRAZINE AND [^{14}C]METHYLAZOXY METHANOL ACETATE BY CULTURED RAT COLON[a]

	pmol DMH-Bound/10 mg DNA	pmol DMH-Bound/10 mg Protein
[^{14}C]-DMH	5000 ± 540	7130 ± 600
[^{14}C]-MAM	1650 ± 50	1920 ± 250

[a] The results represent the mean ± S.E.M. for three experiments. The rat colon was cultured 4 days before addition of either [^{14}C]-DMH (0.26 mM; 3.78 mCi per mmol) or [^{14}C]-MAM acetate (0.26 mM; 39.6 mCi per mmol) and incubated for 8 hr.

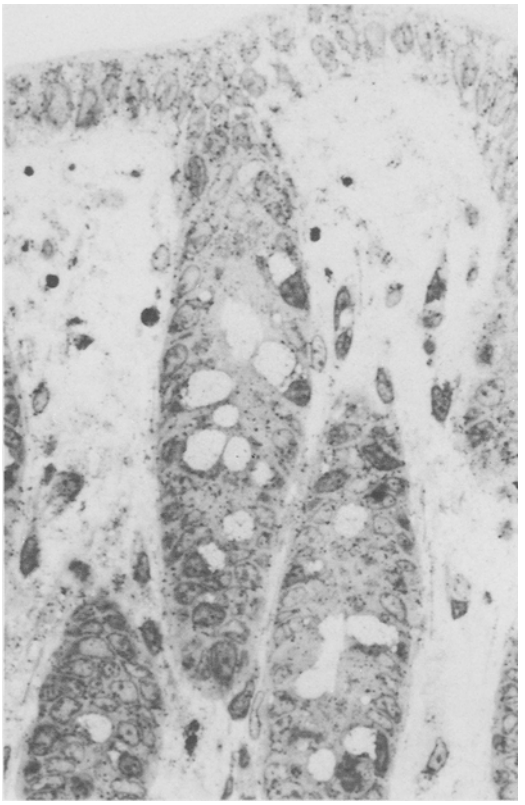

FIG. 9. Autoradiogram. Rat colon was cultured 4 days prior to incubation with [^{3}H]-BP for 24 hr. Toluidine blue. ×540.

the target tissue via the blood circulation (22); (b) the intestinal lumen through deconjugation by the microflora of metabolites formed in the liver (23); and (c) the intestinal mucosa by the mixed function oxidase system (22, 24). The present results show that colonic mucosa can activate several types of procarcinogens into DNA-binding metabolites; some of these procarcinogens have been shown to induce colonic cancer in animal experiments. AFB_1, which produced colonic tumors in rats on a vitamin A-deficient diet (25), had a higher level of binding to DNA than BP or DMBA. Neither BP nor DMBA so far have been shown consistently to induce colonic cancer in rats. However, 3-methylcholanthrene, another PAH, induced cancer of the colon in hamsters (26).

The binding level of 1,2-DMH to DNA, an organotropic carcinogen for the colon in rodents (21), was approximately 15-fold higher than the binding in human colon (Autrup, *published data*) and 20-fold higher than the binding in rat colon of another alkylating agent, DMN, which is not carcinogenic in the large intestine of rodents (21). With similar incubation conditions, the binding level of 1,2-DMH to DNA was 2.5-fold higher than that of MAM acetate, which is an even more potent colon carcinogen in rodent.

The present results indicate that the culture system for rat colon described here is a valuable aid in the study of carcinogen metabolism. The effect of various co- and anticarcinogens on the metabolism of chemical carcinogens by cultured rat colon is presently being investigated (27). Similarly, the results obtained from these experiments can be compared with results obtained from in vivo studies of colon carcinogenesis in the rat (3-5) and with data from carcinogenesis studies in human colonic explant tissues (10).

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The authors would like to thank Dr. Nancy H. Colburn for valuable comments; Steven Fugaro, Nicole Former, and Maria Yamaguchi for technical assistance; and Maxine Bellman for secretarial assistance.