STUDIES ON CARCINOGENESIS WITH 3'-METHYL-4-DIMETHYLAMINOAZOBENZENE AND ANTICARCINOGENESIS WITH METHYLCHOLANTHRENE

by

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REVIEW OF THE LITERATURE

Work on the experimental production of liver cancer in rats originated in Japan in the early 1930's when it became apparent that liver cancer ranked third in cancer frequency among Japanese males.(1) Yoshida, in 1932, was the first to report of the successful experimental production of hepatic carcinoma in rats by feeding them o-aminoazotoluene, a substance used to enhance the yellow color in butter and called "butter yellow".

For the next few years most of the work in this field centered around either the experimental testing of other suspected hepatic carcinogens or studying the effect of various diets on the speed with which liver cancers could be produced using the known carcinogens. Many azo compounds related to the original carcinogenic azo dyes have been studied in an attempt to find a more powerful carcinogen. Most of the compounds tested were found to be less carcinogenic than p-dimethylaminoazobenzene (DAB). One notable exception to this was 3'methyl-4-dimethylaminoazobenzene (m'MeDAB), which was found to be the most powerful hepatic carcinogen.(2)(3) White found a direct relationship between the daily amount of DAB consumed and the incidence of hepatoma.(4) By usage, however, 0.06 per cent dye came to be the standard amount of dye fed, and this greatly facilitated comparison of results. Of the various routes of administration tried, the oral route was found to be the most effective.(5) Rats have been used by most of the experimenters in studying the carcinogenicity of these substances, and Kinosita failed to produce cancer in guinea pigs, rabbits, squirrels, and chickens using DAB.(6) A sex difference was noted by Rumsfeld and co-workers who found that males developed tumors more

rapidly than females when fed m'MeDAB and that castrates held an intermediate position.(7)

Kensler, Dexter, and Rhoads developed the "split-product hypothesis" in which they set forth the belief that it was a breakdown product of the parent dye that was the real carcinogen, rather than the parent dye itself. They found that p-phenylenediamine and N, N-dimethyl-p-phenlenediamine, breakdown products of DAB, were toxic in low concentrations to a fermenting system in which diphosphopyridine nucleotide (DPN) was the limiting factor.(8)(9)(10) Kopac and co-workers also found that N,N-dimethyl-p-phenylenediamine destroyed normal or neoplastic glandular epithelium at concentrations of 0.001 M.(11) Kensler, Dexter, and Rhoads thought that they could predict the carcinogenicity of a methyl derivative of p-DAB by the inhibitory action of its p-diamine split product on a yeast zymase system.(8) This view was challenged by Miller and Baumann who found that many of the compounds that Kensler and his associates had listed as being carcinogenic were either non-carcinogenic, weakly carcinogenic, or else not carcinogenic to the degree that Kensler had reported.(3) The outcome of this is that although some of the split products of the azo dyes are toxic to certain enzyme systems, it is probably the parent azo dye itself which is the carcinogen.

That diet might have something to do with the process of carcinogenesis was first hinted at when Japanese workers found that they obtained delayed tumor formation if liver were added to the usual carcinogenic diet.(12)(13)(14) Later it was found that kidney feeding had a similar but less marked effect.(15)

The same authors found that feeding vitamin B_1 had no effect on the pathologic changes which occurred in the liver, but that yeast had some inhibiting effect on the tumor formation.(16)(17) Other workers subsequently obtained well documented evidence that such things as yeast, wheat, and

wheat bran extract all inhibited tumor formation when added to the carcinogenic diet.[1][18][19][20][21]

Some explanation of why such a diverse group of substances should inhibit tumor formation was offered in 1941 by Kensler and co-workers who discovered partial protection from the effects of the carcinogen could be afforded by adding riboflavin and casein to the diet.[22] Other workers confirmed these results[23][25][24][26][27][28][29][30] and it was established that at least 12 per cent casein[23] and 20 micrograms of riboflavin per gram of food[30] was necessary to obtain the maximum inhibitory effect against the carcinogen.

Numerous other supplements were added to the diets in an attempt to find some basic food substance which would delay or completely inhibit tumor formation. Since rather definite inhibition of carcinogenesis was obtained with riboflavin, it naturally followed that other vitamins fell under scrutiny.[17] The majority of vitamins, including thiamin,[22][24] nicotinic acid,[29] pantothenic acid,[25][29] inositol,[31] folic acid,[29] PABA, and wheat germ oil which is rich in vitamin E,[32] had little or no effect on tumor formation or other pathologic changes in the liver. Contradictory results were obtained when choline, in supplemental amounts, was added to the diet.[23][29] Two authors found that choline had no effect on tumor formation; one[35] thought it and methionine speeded up tumor formation, and another[34] found that it afforded some protection against the preneoplastic changes of necrosis and cirrhosis. These equivocal results are probably best explained by the small number of animals used in some of the experiments, the varying length of time the diets were fed, and the time chosen for killing the animals.

Pyridoxine was found to increase the incidence of tumors, but only slightly.[26][27] Du Vigneaud and his associates reported in 1942 that

biotin exerted a procarcinogenic effect when it was fed with DAB to rats receiving a highly protective diet.[35] Kline and his co-workers substituted egg white for 12 per cent casein and found this diet to be anti-carcinogenic.[36] They proved, however, that this was not due to altered biotin balance by feeding high levels of biotin and getting no reversal of this. Reimann, Stimson, and Medes could find no correlation between the biotin content of the diet and the incidence of tumors.[25] Day and co-workers found that they could increase the incidence of hepatoma from 17 per cent to 78 per cent by adding vitamin B_{12} to a methionine deficient diet containing DAB.[37]

Among other food substances tested, fat fairly consistently accelerated tumor production.[38][39] The type of fat as well as the level of fat in the diet was found to be important. Twenty per cent corn oil produced tumors faster than 5 per cent corn oil,[39][31] but 20 per cent olive oil produced tumors more slowly than 5 per cent olive oil.[31] On the other hand, cottonseed oil, whether at 5, 10, or 20 per cent produced tumors at the same rate. A difference was also noted between coconut oil and corn oil. The fatty acids of hydrogenated coconut oil and the original coconut oil caused retardation or hepatoma development, while the fatty acids of corn oil, and corn oil itself caused an increase in the incidence of tumors.[40] Kline and co-workers postulated that lauric acid, which is present in coconut oil but not in corn oil,[40] might be the critical factor. This, however, appears unlikely in view of the fact that the same authors used mineral oil in place of corn oil and got a decreased incidence of tumors.[41]

Contradictory results were obtained by the addition of the amino acid cystine to the diet with DAB. While some authors found that cystine had no effect on tumor formation,[42][23] others found that a high cystine

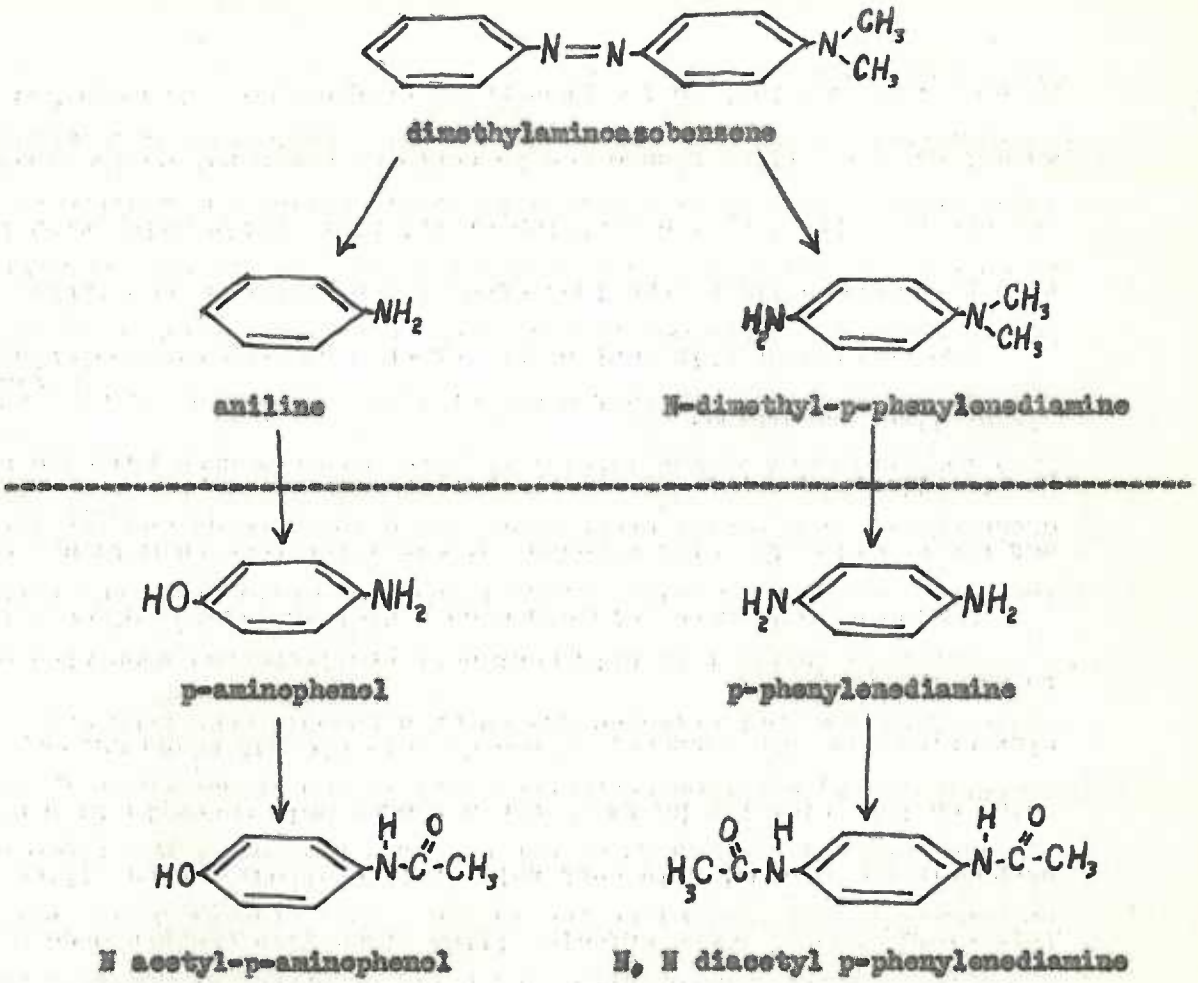
diet (0.5 per cent) would decrease the latent period of tumor formation.[43][33] Gyorgy and co-workers in contradistinction to this found that cystine or choline would enhance the effect of casein in delaying necrosis and cirrhosis of the liver,[34] and Harris found that 1 per cent cystine delayed tumor formation.[29]

Other substances which seemed to decrease the incidence of tumors, or delay their formation to some extent, included Beta sitosterol,[44] 0.05 per cent thiouracil,[31] and detergents.[41] Substances tested and found to have no effect on carcinogenesis were lipocaic,[29] succinic acid[29] and xanthine.[23]

The result of this work on the influence of nutrition on the process of carcinogenesis with DAB can be fairly well summarized by stating that a nutritionally adequate diet with supplements rich in protein and vitamin B complex, especially riboflavin, affords some protection against tumor development.

Since a knowledge of the fate of the carcinogen after it enters a complex biologic system as the rat is essential in studying the mechanism of tumor production, it is not surprising that many workers began investigating the metabolism of m'MeDAB. Stevenson, Dobriner, and Rhoads isolated several breakdown products of DAB in the urine of rats and from this postulated the existence of two other intermediate metabolites.[45]

They presented the scheme on the following page as representing the breakdown of DAB.



The products below the dotted line are the ones which have actually been isolated from the urine. From this, it was thought that the first step in the degradation of DAB was the splitting of the azo linkage.

That DAB might have "labile methyl" groups was suggested by feeding rats DAB in a choline-free diet and finding that this prevented the development of kidney hemorrhages, a characteristic of other compounds containing "labile methyl" groups. It was later shown by Miller and co-workers that DAB and its related compounds could be both methylated and demethylated.(46) When p-dimethylaminoazobenzene was fed to rats, p-dimethylaminoazobenzene, p-monomethylaminoazobenzene, and aminoazobenzene were all found in rat tissue, mostly in the red blood cells.(46)(47)

When p-monomethylaminoazobenzene was fed, all three compounds were found in rat tissue, also indicating that the rat is capable of methylating the mono dye. When p-aminoazobenzene was fed, however, the only dye found was the one fed. These authors by this work also demonstrated that DAB was demethylated *in vivo* prior to reduction of the azo linkage and speculated that the N-dimethyl-p-phenylendiamine step might be skipped. When the methyl group of DAB was labeled with C^{14} and the dye fed to rats, the radioactivity appeared rapidly in the expired CO_2.(48)(49) By forty-eight hours, 50-70 per cent of the C^{14} had been expired as $C^{14}O_2$, 10-30 per cent excreted in the urine,(49) 4-9 per cent excreted in the feces. Some radioactivity of creatinine, choline, and serine was found but only in negligible amounts.(48)(49) By labeling each of the three nitrogens of DAB with isotopic N^{15}, it was found that 90 per cent of the N^{15} was excreted in 72 hours: 70 per cent in the urine, and 20 per cent in the feces.(50) From this study it was determined also that the amount of DAB nitrogen in the liver is very small and that it is greater at 5-6 weeks than at 4 months or one day.

Mueller and Miller obtained evidence that the breakdown of m'MeDAB was of an enzymatic nature in that it was inactivated by heat, it required DPN for activity, and that reduced DPN was unable to reduce the dye.(51) They postulated that demethylase, a riboflavin enzyme which oxidatively demethylates certain N-methyl-L-amino acids may be involved in the demethylation of the azo dyes in the rat liver. These same authors felt that the enzyme responsible for the cleavage of m'MeDAB was most probably a flavoprotein with riboflavinadenine dinucleotide as the prosthetic group.(52) They came to this conclusion after finding that the enzyme system mediating the reductive cleavage of m'MeDAB was largely inactivated by CO_2 and was subsequently reactivated by the specific

addition of riboflavinadenine dinucleotide.[52] Kensler has provided further evidence that riboflavin is implicated in the breakdown of m'MeDAB by showing that rats fed a diet deficient in riboflavin have a decreased amount of riboflavin in their liver and a decreased ability of this liver to destroy m'MeDAB in vitro.[53][54][55] Kensler and his co-workers also found that liver tumor slices from damaged livers resulting from m'MeDAB feeding destroyed less m'MeDAB than did normal liver tissue.[47] This tied in with the above stated fact that the efficiency of liver tissue in destroying the dye was proportional to the riboflavin content in the liver when it was found that the riboflavin content of the liver decreased progressively with continued feeding of the dye,[55][56][57] and that its decrease was roughly proportional to the potency of the carcinogenic dye.[58]

If this is true, that the breakdown of m'MeDAB is mediated through a riboflavin conteining enzyme system, this helps explain the protective action afforded by high riboflavin diets. Riboflavin may participate in the cleavage of the carcinogen to relatively inactive amines such as N, N-dimethyl-p-phenylenediamine and aniline.

Although most of the m'MeDAB fed to rats is demethylated or split at the azo linkage, this is not true of all of the dye as was shown by Miller and Miller when they found aminoazo dyes bound tightly to liver protein.[59][60] Although this dye was found in the liver, it was not found in rat tissues in which m'MeDAB does not induce tumors, as small intestine, kidney, spleen, lung, heart, or skeletal muscle. Neither was dye found in species where m'MeDAB does not form tumors. The amount of dye bound to the liver protein reached a maximum level after 3-6 weeks of m'MeDAB feeding and then decreased until at the time of the appearance

of gross tumors only about half of the maximum level was present in the non-tumorous portions of the liver. This correlates well with the finding that the amount of DAB nitrogen in the liver, as shown by isotopic N^{15} studies, is greatest at this time.(50) No protein bound dye could be detected in the liver tumors. Diets high in riboflavin decreased the amount of protein bound by the azo dyes.

The authors interpreted these results as indicating a qualitative difference between normal liver protein and protein of liver tumors. They suggest that this may be one of the first steps in the carcinogenic process whereby the proteins of the liver are altered in such a way that specific synthetic mechanisms are interfered with, resulting in cells which have completely lost those systems controlling normal growth and hence representing the initial tumor cell.(60)

Sorof and Cohen found the m'MeDAB binding proteins to be in the slow migrating electrophoretic components and found that these were decreased in neoplastic tissue, thus giving support to the theory of Miller and Miller.(60)(62) Hoffman and Schechtman showed that the slow electrophoretic components were also decreased when rats were fedd the non-carcinogenic dye aminoazobenzene and also in 8-18 day old rats. They proposed that this change was most probably a non-specific change associated with rapidly growing tissue and tissue carrying on vigorous protein synthesis.(63)

Further investigation into the intracellular changes which occur during carcinogenesis was made by homogenizing livers of rats fed m'MeDAB and separating various cellular components by differential centrifugation. The fractions studied were the nuclei, the large granules (mitochondria), small granules and supernatant fluid.(64)(65)(66) The protein, DNA, RNA, and riboflavin content were all increased in the nuclear fraction and decreased in the large granule fraction. The small granules showed a decrease in the

protein and RNA, but had the highest concentration of protein-bound dye. The supernatant fluid showed an increase in RNA and a decrease in riboflavin, but also showed the highest concentration of protein-bound dye. When tumor tissue was studied similar changes were found except there was no protein bound dye.(67)

Another step in the process of m'MeDAB breakdown was added when Mueller and Miller discovered that liver homogenates demethylated added m'MeDAB and hydroxylated it to form a new metabolite, 4'hydroxy-4-dimethylaminoazobenzene.(51) Thus, the known metabolic reactions which m'MeDAB was subject to after ingestion by the rat were four in number: 1.) Reductive cleavage of the azo linkage, 2.) demethylation of the N-Methyl groups, 3.) hydroxylation of the 4' position, 4.) the combination of the methylated parent compound with liver protein.(68)

The theory that a normal cell might be converted to a cancer cell by the depletion of enzymes is, of course, an attractive theory and one that has stimulated much investigation. Many studies were undertaken to determine which substances became depleted in the liver and which enzyme underwent a decrease in their activity during the process of carcinogenesis from feeding m'MeDAB was fed.(54)(55)(56)(57)(58) This decrease was found to be roughly proportional to the potency of the carcinogenic dye used.(58) Kensler and his co-workers found that DPN (Coenzyme I) was decreased 60 per cent in the liver of animals fed m'MeDAB and that some of the breakdown products of m'MeDAB were toxic in low concentration to a fermenting system in which DPN was the limiting factor.(55)(8)(9)

Nakatani and co-workers reported an increase in the anaerobic glycolysis as m'MeDAB was fed and a sudden increase in aerobic glycolysis with hepatoma formation.(69) Orr and Strickland, on the other hand,

observed no change in the glycolytic metabolism of liver tissue in the precancerous phase.[70] They found, however, that tumors produced by m'MeDAB showed a qualitative difference in metabolism from normal or cirrhotic liver in that the substrate for glycolysis was glycogen in liver tissue and glucose in the tumors.[70]

The desoxyribonucleic acid was found to increase progressively as m'MeDAB was fed.[71][72][73][74][56] Ribonucleic acid showed this same trend.[73] This is not surprising in view of the increased mitotic activity stimulated by the m'MeDAB, particularly in the cancerous stage.

Other enzymes whose activities have been reported as being decreased include glyoxalase,[75] catalase,[71] xanthine oxidase,[76] several sulfhydryl enzymes,[77][78] histidase,[79] arginase,[79] succinoxidase[80][81][72][71] DPN-cytochrome C reductase,[72] and cytochrome oxidase.[71] These have been pure empirical observations and though theories abound, no causal relation has been demonstrated between the reduction in the activity of these enzymes and the process of carcinogenesis.

Another phase of carcinogenesis that has been investigated is that of inhibition of hepatoma formation. The effect of diet on the rate of growth of hepatic tumors has already been discussed. Methylcholanthrene, a potent carcinogen in its own right, has been shown to inhibit the formation of m'MeDAB induced liver tumors. Richardson and Cunningham[82] were the first to report on this inhibitory action of MCA. Since their first report, this has been confirmed and elaborated upon by other investigators.[83][84] The mode of action of this inhibition has not been determined. That MCA might act by suppression of tissue growth has been shown by White,[85] who slowed the growth of rats by feeding them MCA and by Hertz who inhibited comb growth in chicks by topical application of

MCA.[86] Strong suggested that MCA might cause somatic mutation when he got thirteen mutations in coat color in mice after treating them with MCA.[87]

Nitrogen mustard has also been found to inhibit m'MeDAB induced tumors.[88][89] Griffin's studies indicated that Nitrogen mustard might act by blocking mitosis and the synthesis of DNA.[88]

PURPOSE

The purpose of this experiment was threefold:

1. To establish the minimal length of time that m'MeDAB must be fed to rats to produce liver cancer. In conjunction with this the pathologic changes which occur in the liver prior to the formation of cancers were to be studied.

2. To make further studies into the inhibition of m'MeDAB induced tumors by the use of MCA. It has been shown by previous investigation in this laboratory that the addition of MCA to a basic diet containing the carcinogen m'MeDAB prevented the formation of tumors ordinarily produced when m'MeDAB alone was fed. The purpose of this phase of the experiment was to see if pretreatment with MCA had any influence on tumor incidence when m'MeDAB was subsequently fed.

3. To determine if any correlation existed between the pathologic state of the liver and the amount of m'MeDAB it could destroy <u>in vitro</u>.

MATERIALS AND METHODS

Animals:

Five hundred and twenty-eight albino rats of the Sprague-Dawley strain were used. There were 258 males and 270 females. During the experiment these animals were housed in individual wire cages and were given water ad lib from drip bottles.

Groups:

Two hundred and sixteen animals were used for the experiment in which the basal diet and m'MeDAB were fed. All of these animals were started at the same time on a basal synthetic diet to which 0.06 per cent m'MeDAB had been added. At two week intervals, starting with the second week, a group of animals (Groups A to I) were taken off m'MeDAB and maintained on the basal diet for the rest of the experiment as shown in Table I. At the end of 18 weeks all of the animals still alive were on the basal diet. After a group of animals had been on the basal diet for two weeks four of the animals were killed and two to four were killed every two weeks until all the animals in that group were gone. The killing of the animals was staggered in this manner so that the sequence of pathological change in the liver could be followed up to the point of cancer formation.

Two hundred and seventy animals were used for the mixed feeding of m'MeDAB and MCA in the basal diet. These animals were divided into three groups of 90 animals per group. Each group contained three series of 30 animals per series.

The first group (Group K) was started on the basal diet to which 0.06 per cent m'MeDAB had been added. Three series of thirty animals

each were then taken off the m'MeDAB at 8, 10, and 12 weeks respectively, and fed the basal diet containing 0.0067 per cent MCA for the remainder of the experiment as shown in Table II. It was intended that four animals from each series be killed after they had been on the diet containing the MCA for four weeks, and four animals from each series be killed every subsequent four weeks for the remainder of the experiment. The animals died so rapidly, however, that this schedule could not be adhered to.

The second group (Group L) was started on the basal diet to which both 0.06 per cent m'MeDAB and 0.0067 per cent MCA had been added. Three series of thirty animals each were then taken off the diet containing both the m'MeDAB and the MCA at 5, 7, and 9 weeks respectively, and were maintained on the basal diet containing only the 0.06 per cent m'MeDAB. Four animals from each series were killed every four weeks after they were on the diet containing only the m'MeDAB as shown in Table II.

The third group (Group M) was started on the basal diet to which 0.0067 per cent MCA had been added. Three series of thirty animals each were then taken off the MCA at 5, 7, and 9 weeks respectively, and fed the basal diet containing 0.06 per cent m'MeDAB for the remainder of the experiment. Four animals from each series were killed every four weeks after they were on the diet containing 0.06 per cent m'MeDAB, as shown in Table II.

Forty-eight control animals were used for this phase of the experiment. Twelve animals were on basal diet containing 0.06 per cent m'MeDAB; twelve animals were on basal diet containing 0.0067 per cent MCA; twelve animals were on basal diet containing 0.06 per cent m'MeDAB plus 0.0067 per cent MCA; and twelve animals were on the basal diet alone. The animals were maintained throughout the experiment on the

original diet started. As noted in Table II, several of these animals were not autopsied. This was because of cannibalism which resulted unless the animals were removed from their cages immediately after death.

Diets:

The basal synthetic diet used in this experiment was one formulated by Griffin.(56) It consists of the following:

Casein	18%
Glucose Monohydrate	73%
Corn Oil	5%
Salt Mixture	4%
Containing NaCl, $Ca_3(PO_4)_2$, $MgSO_4$, KCl, FeSi, NaF, KI, $CuSO_4$, KH_2PO_4, $K_2Al_2(SO_4)_2$.	

Thiamin	60 mg/20Kg.
Riboflavin	40 mg/20Kg.
Pyridoxine	50 mg/20Kg.
Calcium Pantothenate	140 mg/20Kg
Choline	10 gms/20Kg.

The 0.06 per cent m'MeDAB was made by adding 6.0 Gms. of m'MeDAB to 10 Kg. of the basal diet.

The 0.0067 per cent MCA was made by adding 200 mg. of MCA to 3,000 Gms. of the basal diet. No more than 3,000 Gms. were mixed at one time to insure a homogeneous mixture.

The 0.06 per cent m'MeDAB plus 0.0067 per cent MCA was made by adding the 200 mg. of MCA to 3,000 Gms. of 0.06 per cent m'MeDAB rather than the basal diet.

Technique Of Autopsies:

Autopsies were performed and liver tissue saved from all animals whether they were killed or whether they died in their cages. Most of the animals were killed by putting them in a jar saturated with ether.

but some of the animals used in the metabolism experiment were killed by a blow on the head.

The liver was removed from the animal and notes were made on its general appearance and any pathology present. Four or five representative sections were taken from various portions of the liver and transferred to Vandegrift's Fixative. (90)

Vandegrift's Fixative:

Ethyl Alcohol 95%	80.0 cc.
Formalin, Full Strength	12.0 cc.
Glacial Acetic Acid	4.5 cc.
Picric Acid	4.0 Gms.
Mercuric Chloride	0.2 Gms.
Urea	0.5 Gms.

All tissue sections were stained with Eosin, Orange G., and Hematoxylin.

Liver Slice Technique:

Rats used for the liver slice experiment were obtained from the L and M groups of animals. Early in the experiment these rats were killed with ether like the other rats, but later it was felt that better results were obtained if the rats were killed with a blow on the head. The liver was removed from the animal immediately while the heart was still beating and four sections of liver were taken for slicing. These sections were placed on top of a jar filled with ice water used to forestall autolysis as much as possible. Two strips of cover glass 1 cm. wide and 4-5 cm. long were fixed to an ordinary glass slide 1 cm. apart. The depression between these strips of cover glass allowed slices of liver approximately 0.5 mm. thick to be cut when the glass slide was pressed against the section of liver and a razor blade held with a hemostat was drawn along the two strips of cover glass. After enough slices of liver to make 150-200 mg. were obtained, the remaining section of liver was placed in a separate labeled bottle of fixative so that microscopic sections could

be made. The liver slices were placed in a pre-weighed 25 ml. flask containing 1.9 ml. Ringer Phosphate solution (pH 7.4) and re-weighed so that the exact weight of the liver slices could be obtained. One-tenth mg. of m'MeDAB in 0.1 ml. of 95% ethyl alcohol was then added to the flask and it was placed in the water bath. Three of the four flasks containing the liver slices from one animal were treated in this fashion. To the fourth flask 1 ml. of 20 per cent trichloroacetic acid in 1:1 acetone-ethanol was also added to kill the liver tissue before incubation. This was used as a control to determine what proportion of the m'MeDAB added could be recovered.

The tissue slices were incubated aerobically in the water bath at 37.5 C under constant agitation for 90 minutes. At the end of 90 minutes the flasks were removed from the water bath and 1 ml. of 20 per cent trichloroacetic acid in 1:1 acetone-ethanol was added to kill the tissue and stop the breakdown of m'MeDAB.

The tissue was then transferred to a glass homogenizer with the aid of 2 ml. of 20 per cent trichloroacetic acid solution and homogenized. The homogenate obtained was transferred to a centrifuge tube with the aid of a small amount of the 20 per cent trichloroacetic acid solution and water (1:1), and centrifuged at 2500 R.P.M. for approximately 10 minutes. The supernatant fluid was decanted into a 25 ml. volumetric flask. Another small amount of the 20 per cent trichloroacetic acid solution and water (1:1) was added to the precipitate, mixed thoroughly, and recentrifuged. The supernatant from this was poured into the same 25 ml. volumetric flask with the first portion. The contents of this 25 ml. volumetric flask were then diluted to the mark with the trichloroacetic acid solution and water (1:1). Two ml. of this solution

were put in a 25 ml. Erlenmeyer flask containing 6 ml. of trichloroacetic acid solution and water (1:1), making a colored solution sufficiently dilute to be read with a spectrophotometer. The per cent transmittance of the solution was read spectrophotometrically, using a wave length of 525 milimicra. The micragrams of m'MeDAB per ml. of solution was then read from a curve made by plotting the per cent transmittance of various concentrations of m'MeDAB in trichloroacetic acid solution and water (1:1).

TABLE I

Group	Number of Animals	Diet	Animals Died	Animals Killed
A	40	m'MeDAB 2 weeks Basic until autopsy	2	38
B	36	m'MeDAB 4 weeks Basic until autopsy	4	32
C	32	m'MeDAB 6 weeks Basic until autopsy	8	24
D	28	m'MeDAB 8 weeks Basic until autopsy	5	23
E	24	m'MeDAB 10 weeks Basic until autopsy	7	17
F	20	m'MeDAB 12 weeks Basic until autopsy	5	15
G	16	m'MeDAB 14 weeks Basic until autopsy	4	12
H	12	m'MeDAB 16 weeks Basic until autopsy	5	7
I	8	m'MeDAB 18 weeks Basic until autopsy	4	4

TABLE II

Group	Number of Animals	Diet	Animals Died	Animals Killed
K_1	30	m'MeDAB 8 weeks (7 died before 8 weeks) MCA until autopsy	25	5
K_2	30	m'MeDAB 10 weeks (13 died before 10 weeks) MCA until autopsy	30	0
K_3	30	m'MeDAB 12 weeks (10 died before 12 weeks) MCA until autopsy	30	0
K_4 (Controls)	12	m'MeDAB until autopsy (4 not autopsied)	11	1
L_1	30	m'MeDAB + MCA 5 weeks m'MeDAB until autopsy	10	20
L_2	30	m'MeDAB + MCA 7 weeks m'MeDAB until autopsy	16	14
L_3	30	m'MeDAB + MCA 9 weeks m'MeDAB until autopsy	18	12
L_4 (Controls)	12	m'MeDAB + MCA until autopsy (3 not autopsied)	5	7
M_1	30	MCA 5 weeks m'MeDAB until autopsy	6	24
M_2	30	MCA 7 weeks m'MeDAB until autopsy	7	23
M_3	30	MCA 9 weeks m'MeDAB until autopsy	9	21
M_4 (Controls)	12	MCA until autopsy (1 not autopsied)	5	7
0 (Controls)	12	Basic until autopsied	0	12

OBSERVATIONS AND RESULTS

Groups A through I:

This group of 216 animals was fed m'MeDAB for from two to eighteen weeks, maintained thereafter on basic diet, and killed at intervals so that the pathologic changes in the liver could be observed. As can be seen from Chart 1, minimal cirrhosis appeared after only two weeks on m'MeDAB in 26 per cent of the animals. The amount and severity of the cirrhosis increased progressively after this until by the eighteenth week of feeding m'MeDAB 82 per cent of the animals had severe cirrhosis. After eight weeks on the dye, 100 per cent of the animals had cirrhosis of varying degrees.

The grading of the cirrhosis as minimal, moderate and marked was, of course, arbitrary and each classification covered a rather wide range of liver damage. A liver was considered to have minimal cirrhosis when the connective tissue proliferation was confined to the portal spaces with some extension between the lobules (Figure 1). Moderate cirrhosis was an extension of this same process to often times completely surround a lobule. There was generally an increase in connective tissue throughout all areas of the liver but without too much disruption of liver architecture (Figure 2). In marked cirrhosis the connective tissue had invaded the liver lobule and distorted the liver architecture, many times to the extent that true liver lobules could not be distinguished (Figure 3). In general, both the quantitative and qualitative approaches were used in all three groups in an attempt to separate them. In actual practice it was found impossible to adhere strictly to these criteria because of variations between lobes in the same liver and even variations

between different areas in the same lobe. It was occasionally necessary, therefore, to use such terms as "minimal to moderate" and "moderate to marked" when describing the changes seen in these livers.

Chart 1 summarizes the findings in regard to cirrhosis in this group. The degree of cirrhosis in the liver usually correlated well with the amount of damage in that liver, but this was not always true. Other pathological changes occurring along with the cirrhosis were bile duct proliferation, bile duct cysts, fatty metamorphosis, nodular hyperplasia, and cholangiofibrosis. In most cases these occurred commensurate with the amount of cirrhosis, becoming progressively more severe as the cirrhosis became more severe. Occasionally, however, a liver with only moderate cirrhosis would have large sections of it destroyed by innumerable bile duct cysts, or massive cholangiofibrosis.

Further pathological description of these ancillary changes should be given at this time.

Bile duct proliferation consists merely of an increase in the number of bile ducts normally found in the portal spaces. This increase may vary from only a few bile ducts to hundreds of bile ducts. These frequently become filled with fluid, expand, and form bile duct cysts. The lumen of these are many times the size of a normal bile duct lumen and are lined with a single layer of cuboidal bile duct epithelial cells. The liver parenchyma surrounding them is sometimes compressed and if the cysts are massive, they may greatly alter the liver pattern (Figure 5).

Fatty metamorphosis consists of fat globules within the liver cells which distend them. This occurred usually in isolated cells throughout the liver lobule, but occasionally was more concentrated in restricted areas of the liver (Figure 6).

Nodular hyperplasia has been designated by various other names,

such as benign hepatoma, but in as much as it most probably represents a regenerative attempt on the part of the liver, we prefer the term nodular hyperplasia. These are well circumscribed areas surrounded by varying amounts of connective tissue. The cells are larger and paler staining than normal liver cells but their pattern of distribution is orderly and not unlike that of an ordinary liver lobule (Figure 7).

Cholangiofibrosis represents a rather marked departure from anything seen in a normal liver. The basic pattern is that of a marked proliferation of bile ducts surrounded by swirls of connective tissue which crowd and distort the bile ducts. The bile ducts are filled with an eosinophilic material and frequently many polymorphonuclear leukocytes and other signs of acute inflammation. The bile duct cells are usually darker staining than normal and they assume more bizarre shapes than normal cells. This cholangiofibrosis may involve an area as small as a high power or as large as an entire lobe (Figure 4).

Bile duct proliferation and bile duct cysts occurred uncommonly after two or four weeks on m'MeDAB, but became common after six weeks on the dye. This was also true of the other changes of fatty metamorphosis, nodular hyperplasia, and cholangiofibrosis, although the latter did not become marked until eight to ten weeks.

The incidence of tumors found in these animals is represented graphically in Chart 2. This experiment showed that it was necessary to feed the animals m'MeDAB for a ten week period before tumors occurred. In addition to this, it was necessary that the animal live twenty weeks after m'MeDAB was first fed before tumors began to appear. Only one animal with a tumor lived less than twenty weeks and this one was on m'MeDAB for eighteen weeks.

The reason for the discrepancy in the G and F groups is not readily

apparent. There were 18 animals in the F group and 14 animals in the G group. Of these, 8 in the F group developed tumors whereas only 4 in the G group developed tumors.

The liver tumors morphologically were basically of two types, malignant hepatomas and adenocarcinomas. Many other classifications have been proposed, but even this one seems somewhat arbitrary since both types of tumors were frequently found in the same animal and many times one type was seen to merge into the other.

The hepatomas were not unlike normal liver tissue. The cells were more basophilic, hyperchromatic, and varied more in size and shape, but the basic architectural pattern was not radically different from that of normal liver. There were usually enough neoplastic characteristics to these tumors, however, that little trouble was encountered in distinguishing them from the normal or regenerating liver tissue (Figure 8).

The adenocarcinoma type of tumors consisted of cuboidal or columnar epithelium arranged in acini and surrounded by varying amounts of connective tissue. These frequently became acutely infected. These were readily distinguishable from non-neoplastic liver tissue (Figure 9).

Groups M and L:

Group M consisted of 90 animals which had been fed a basic diet containing 0.0067 per cent MEA for a period of 5 to 9 weeks and then fed m'MeDAB for the rest of the experiment. The L group consisted of 90 animals which had been fed the basic diet containing both 0.06 per cent m'MeDAB and 0.0067 per cent MEA for a period of 5 to 9 weeks and then maintained on basic diet containing only m'MeDAB for the remainder of the experiment.

As shown by the accompanying graph (Chart 3) the pretreatment with MEA did lower the incidence of hepatic tumors within the length of time

the experiment was conducted. It did not, however, prevent the formation of tumors. The animals represented on the graph as being on m'MeDAB only are from a previous experiment and are used as the standard against which the animals from this experiment are compared.

The 90 animals in group M had been divided into thirds and placed on MCA for 5, 7, and 9 weeks. In order to enlarge the groups used in the graphs, these were combined into one group and all were considered to have been on MCA for 7 weeks. The same thing was done with the animals in group L and all animals were considered to have been on MCA plus m'MeDAB for 7 weeks.

Cirrhosis and other structural changes in the liver developed at essentially the same rate and in the same sequence as in the A to I groups previously discussed. The only difference was that in the M and L groups a marked proliferation of bile duct cells occurred, a condition not seen or seen only to a minimal degree in the A to I groups. This proliferation of bile duct cells began to appear at 4 weeks, reached a maximum between 8 and 12 weeks, and then decreased until it was almost entirely absent by the time the most advanced stages of cirrhosis were reached.

Morphologically this bile duct cell proliferation consisted of an increase in bile duct cells which did not form bile ducts but rather extended from the portal areas, surrounded the lobules and infiltrated into the periphery of the lobule. As the more advanced stages were reached, the bile duct cells infiltrated deeper into the lobule (Figures 10 and 11). This accounted for the majority of the deaths that occurred in the animals which had been on m'MeDAB only a short time. The animals that survived this period of marked bile duct cell proliferation and infiltration showed a progressive decrease in the number of bile duct cells as cirrhosis began to get more marked.

The K animals were not included in the graph comparing the incidence of tumor formation. The reason for this was that these animals began dying three weeks after being placed on the m'MeDAB diet and the longest any of them lived was 16 weeks. Only five of the animals in this group were killed, the rest died. In most of the cases there was enough damage to the liver either by the bile duct cell infiltration or cirrhosis to account for their deaths.

Twelve animals were placed on the basic diet and maintained on this throughout the experiment as controls on the adequacy of the basic diet. When these animals were autopsied, all were found to have normal livers.

Twelve animals were put on MEA at the start of the experiment as controls against any liver damage that MEA might produce. Eleven of these were autopsied and all had normal livers.

Twelve animals were maintained on MEA plus m'MeDAB as controls for this diet. Two of these animals killed after five weeks showed minimal cirrhosis and minimal bile duct cell proliferation. Seven killed after being on this diet for 28 to 34 weeks showed only moderate to marked cirrhosis; none had cancers. Three animals did not reach autopsy due to cannibalism.

Twelve animals were maintained on a diet containing m'MeDAB throughout the experiment. Of the seven animals which reached autopsy, all had massive liver cancers.

Liver Slice Incubation:

The results of this part of the experiment were disappointing. Four slices from each liver were incubated, three of these were living tissue, and one was killed by the addition of trichloroacetic acid prior to incubation. The killed tissue was to serve as a control on the amount of m'MeDAB that was lost in the process of extraction. Although the

morphologic changes in the slices taken from the same liver were essentially the same, there was a marked variation in the amount of m'MeDAB the slices destroyed. No consistent difference existed between the amount of m'MeDAB destroyed by normal liver tissue and pathologic tissue. Also, while in the majority of the cases the amount of m'MeDAB extracted from the killed sample was greater than that extracted from the samples of viable liver, there were enough exceptions to this to cast doubt on the validity of the others. The results in general were too inconsistent to allow any definite conclusions to be drawn.

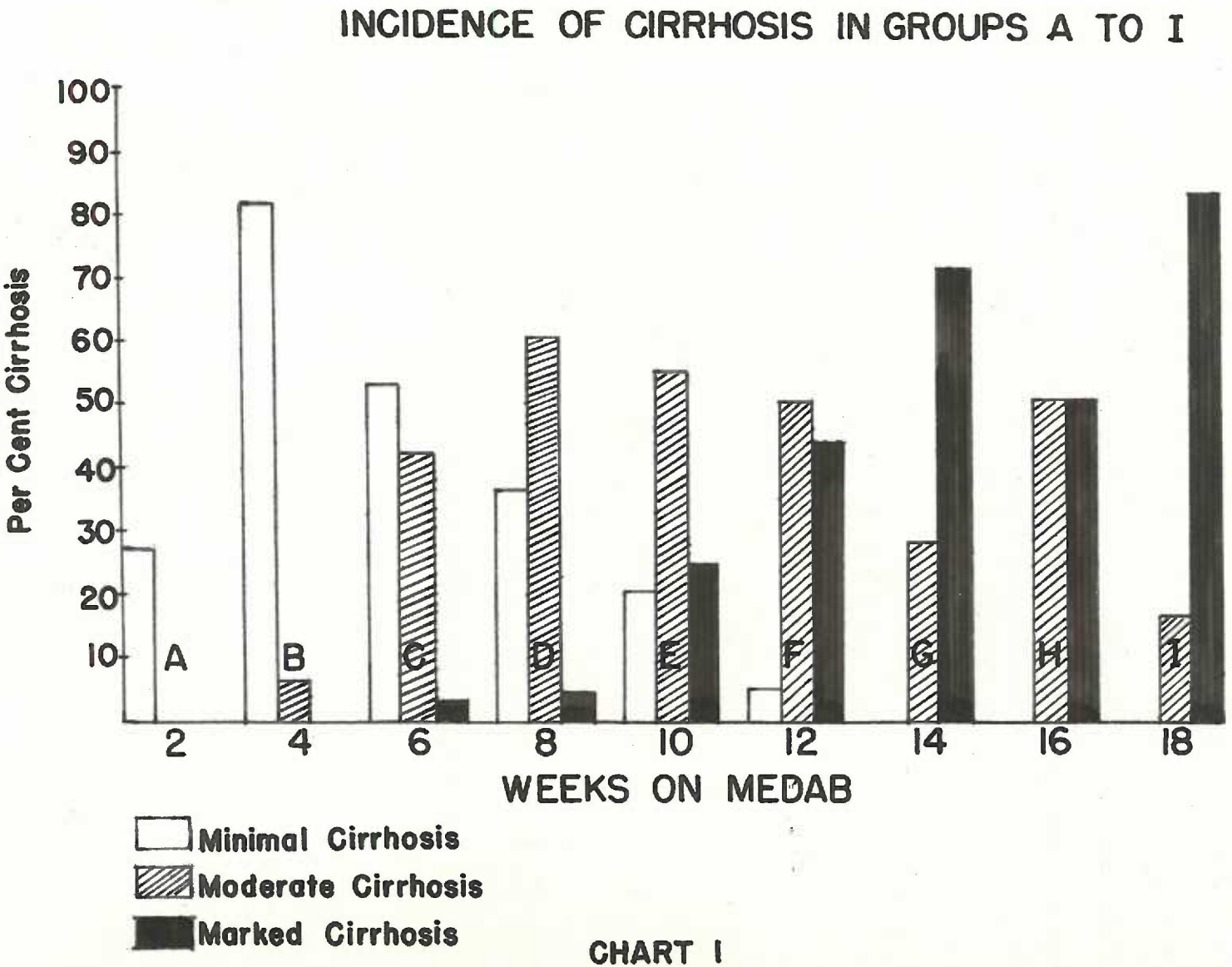

INCIDENCE OF CIRRHOSIS IN GROUPS A TO I
Per Cent Cirrhosis
100
90
80
70
60
50
40
30
20
10
A
B
C
D
E
F
G
H
I
2
4
6
8
10
12
14
16
18
WEEKS ON MEDAB
Minimal Cirrhosis
Moderate Cirrhosis
Marked Cirrhosis

CHART I

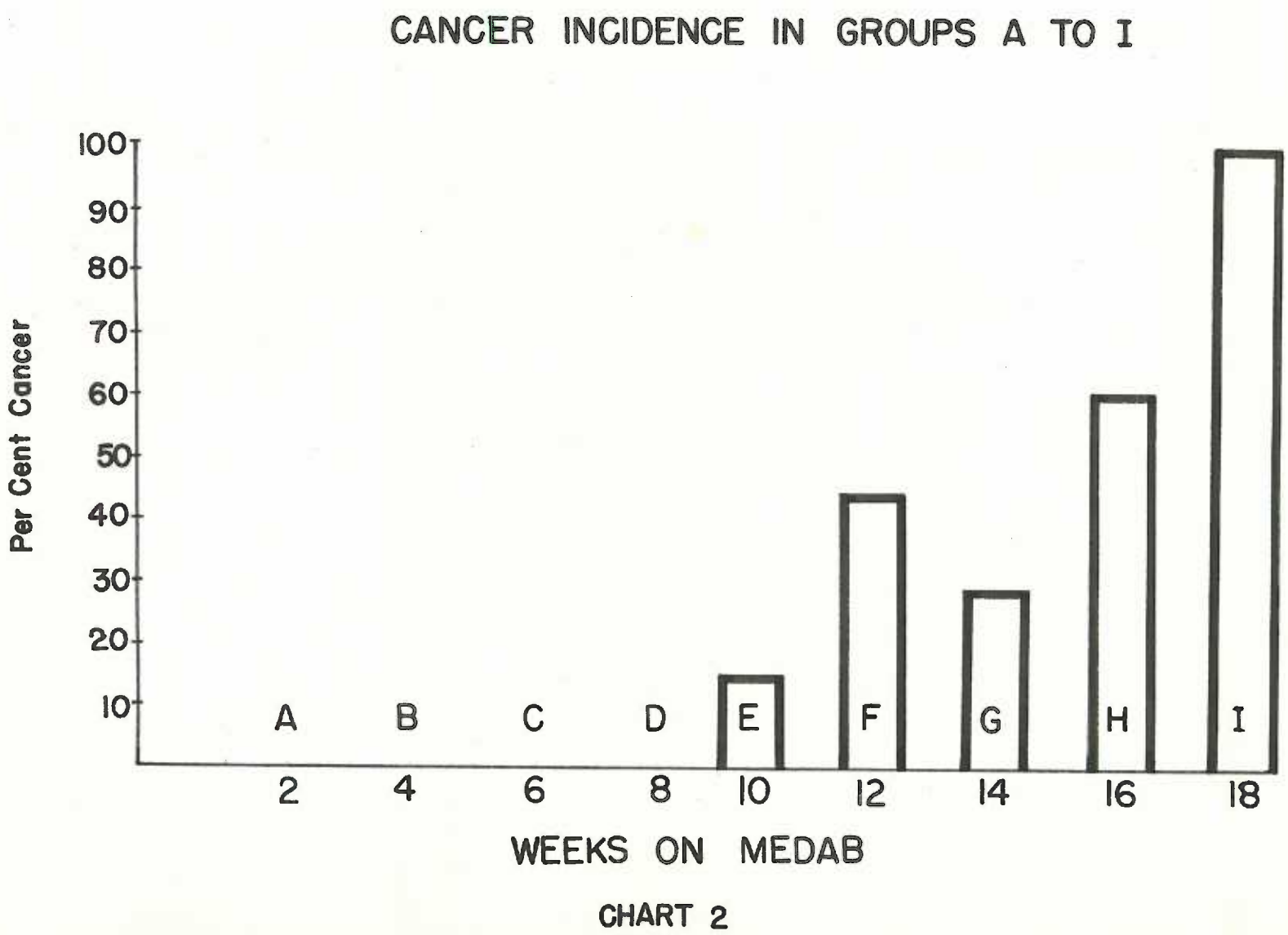

CANCER INCIDENCE IN GROUPS A TO I
Per Cent Cancer
100
90
80
70
60
50
40
30
20
10
A
B
C
D
E
F
G
H
I
2
4
6
8
10
12
14
16
18
WEEKS ON MEDAB

CHART 2

CUMULATIVE PERCENTAGE OF ANIMALS WITH CANCER

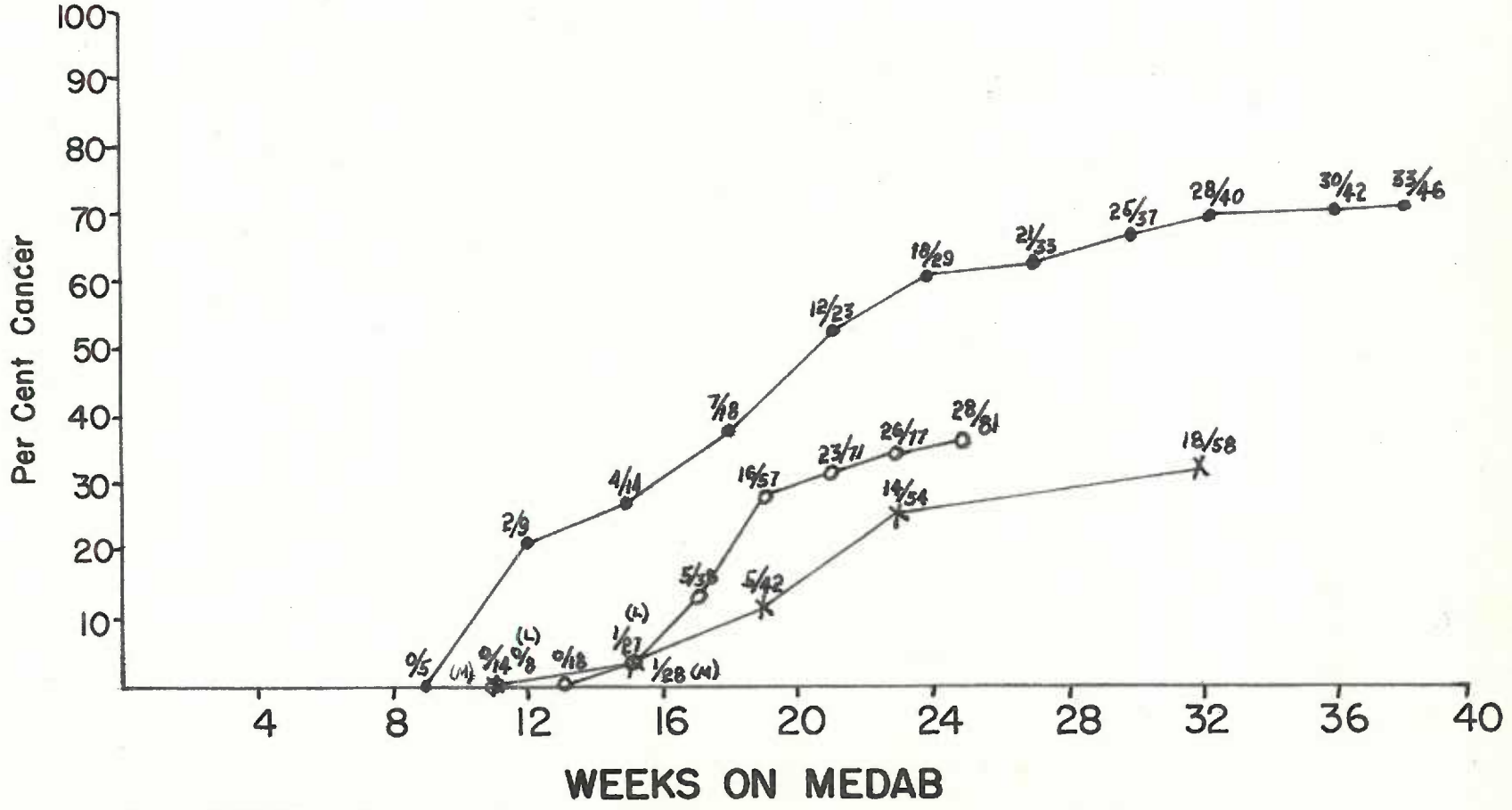


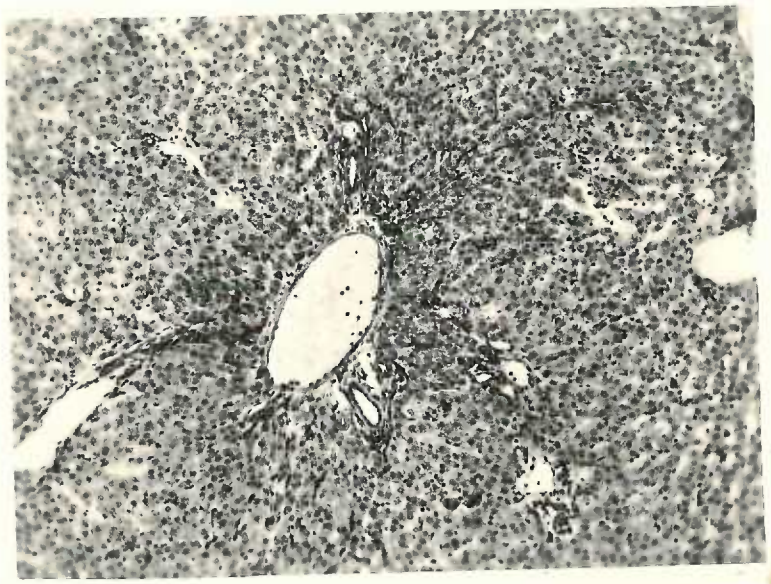
CHART 3

Figure 1:

Minimal cirrhosis in a rat on m'MeDAB for 6 weeks and basic diet for 4 weeks.

Figure 2:

Moderate cirrhosis in a rat on MCA for 9 weeks and m'MeDAB for 30 weeks. This rat had marked cirrhosis in most of the liver, but there were also areas only moderately involved.



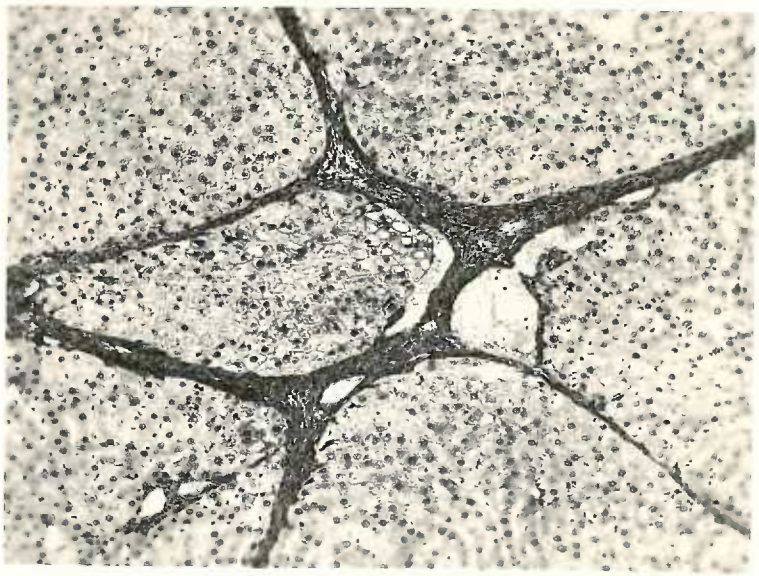
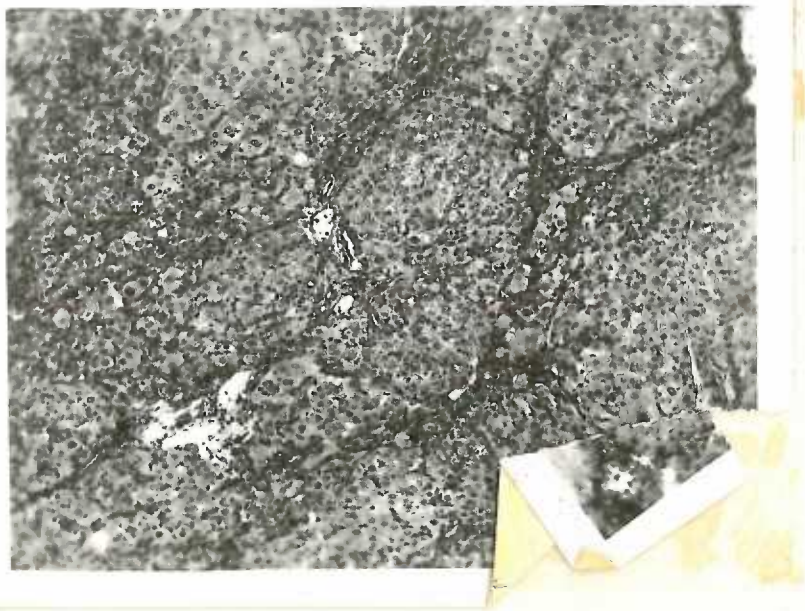


Figure 3:

Marked cirrhosis in a rat on MCA for 7 weeks and m'MeDAB for 16 weeks.

Figure 4:

Area of cholangiofibrosis in a rat on MCA for 5 weeks and m'MeDAB for 16 weeks. This rat had moderate cirrhosis.



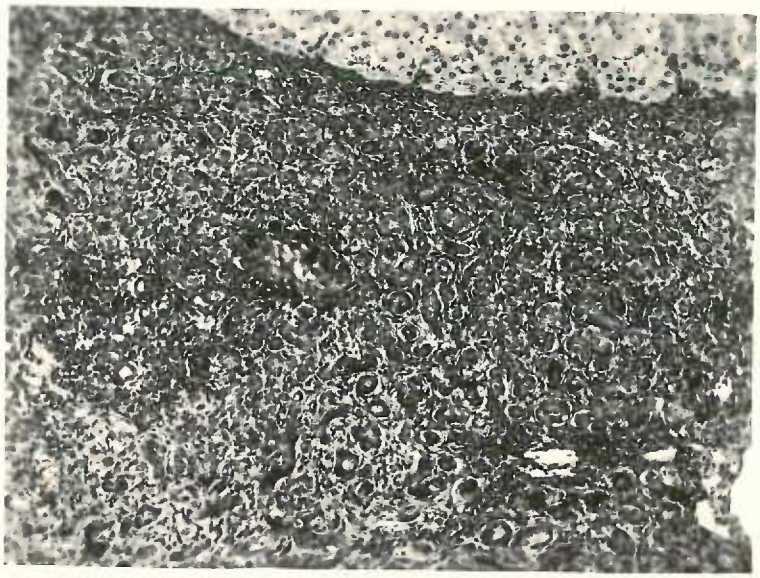


Figure 5:

Area of bile duct proliferation in lower half of photograph and bile duct cysts in upper half. Rat on MEA plus m'MeDAB for 9 weeks and m'MeDAB for 12 weeks.

Figure 6:

Example of fatty metamorphosis in a rat on m'MeDAB plus MEA for 9 weeks, and m'MeDAB for 8 weeks.

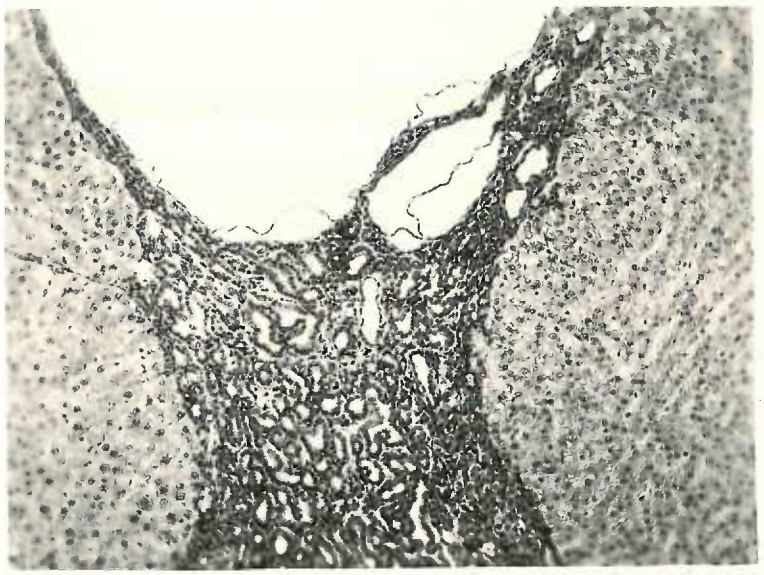
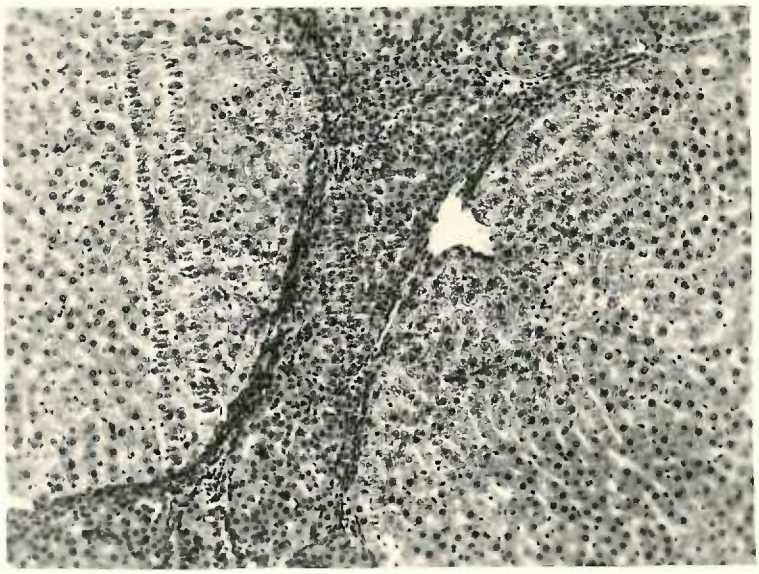


Figure 7:

Nodular hyperplasia in a rat on m'MeDAB for 12 weeks and basic diet for 6 weeks. Two hyperplasic nodules are separated by a strip of relatively normal liver tissue.

Figure 8:

Malignant hepatoma in a rat on MEA for 5 weeks and m'MeDAB for 24 weeks.



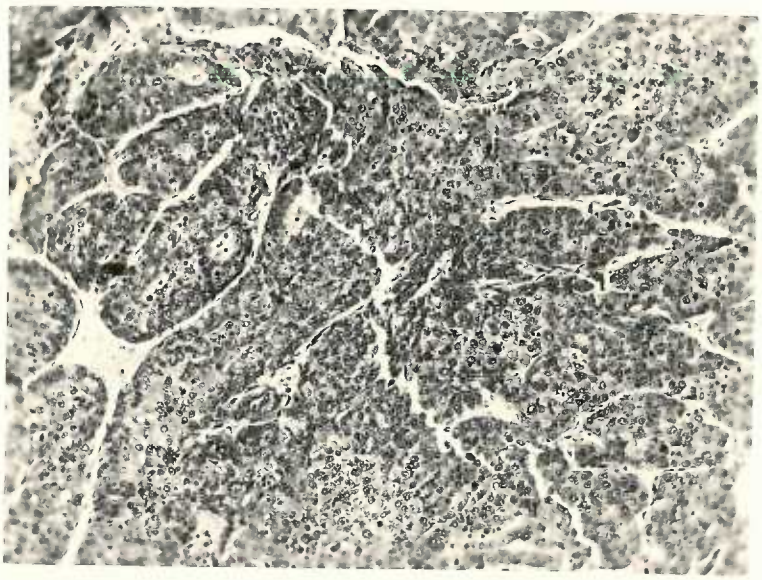
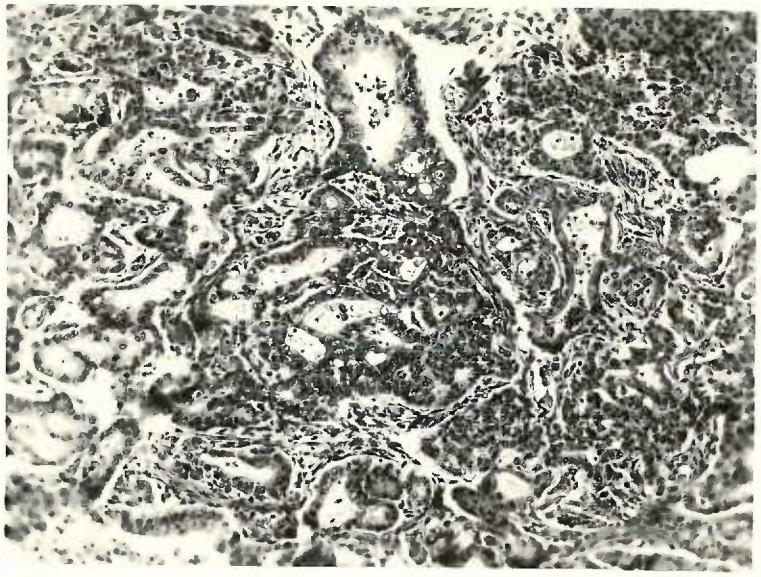


Figure 9:

Adenocarcina of the liver in a rat on MEA for 9 weeks and m'MeDAB for 20 weeks.

Figure 10:

Moderate bile duct cell proliferation in a rat on m'MeDAB plus MEA for 5 weeks and m'MeDAB for 4 weeks.



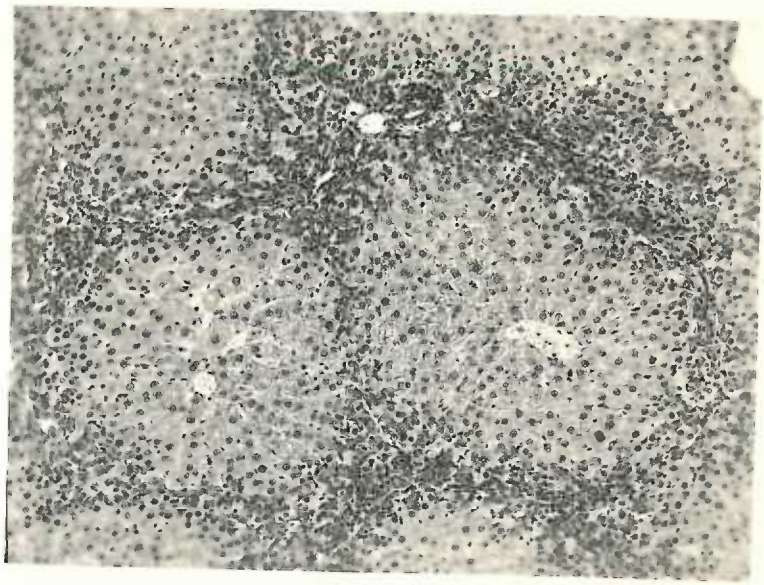


Figure 11.

Marked bile duct cell proliferation and infiltration on m'MeDAB plus MEA for 7 weeks and m'MeDAB for 12 weeks.

DISCUSSION

The experiment in which m3MeDAB was fed to rats for varying lengths of time showed that the rats must be exposed to the carcinogenic dye for a minimum length of time before tumors develop. This minimum length of time was found to be ten weeks. An additional ten weeks, making a total of twenty weeks from the time the dye was first fed, was found to be necessary before tumors began to appear. This agrees, for the most part, with the work of Certelli (91) who found that it was necessary to feed 0.05 per cent m'MeDAB for 69 days to produce tumors. However, she began to observe tumors by abdominal palpation as early as thirteen, sixteen, and eighteen weeks after first feeding the dye. This discrepancy is probably accounted for by the fact that she was using a diet low in protein and riboflavin, a condition known to speed up tumor formation.

The sequential pathologic changes that occur in the liver on continuous feeding of the azo dye carcinogens have been well worked out by Orr (92), Opie (93), Edwards and White (94), and Price and co-workers (95). The changes found in this experiment agree essentially with these found by these authors with one exception to be discussed later.

Many types of tumors have been described as occurring in livers of rats fed azo dyes.

Orr classifies the tumors as bile duct carcinoma (cholangioma), bile duct cystadenoma, and liver cell carcinoma. His bile duct carcinoma corresponds to what we called cholangiofibrosis, a condition which we consider to be benign. This condition was considered to be benign because it may occur very early, it was not found to metastasize, it occurs in multiple foci throughout the liver, and it grows more slowly than the tumors

considered to be malignant. What Orr calls bile duct cystadenoma we have called bile duct proliferation and bile duct cysts. In only one case did this have the suggestion of malignancy. Orr groups as liver cell carcinoma the only two tumors that were considered to be malignant in this paper.

Opie lists four tumors as occurring in the livers of rats fed the azo dyes, namely trabecular hepatomas, adenohepatomas, cystadenomas, and cholangiomas, but states that a large part of these tumors should be regarded as benign. The trabecular hepatomas correspond to the malignant hepatomas in this paper, and the adenohepatomas correspond to the adenocarcinomas. The cystadenomas Opie describes are the same as the bile duct cystadenoma described by Orr, and bile duct proliferation and cysts described in this paper and considered benign with one possible exception. The cholangiomas were the same as the cholangiofibrosis in this paper.

The classification of Edwards and White is the same as the one used in this paper except that they further classify the hepatomas as to Type I and Type II. Although the characteristics described for the Type I and Type II hepatomas were also seen in the animals in this experiment, it was not felt that these tumors presented enough morphologic dissimilarity to warrant separate classification.

Price and co-workers classification of hepatomas, cholangiomas, and mixed tumors also corresponds with the one used in this paper, the cholangiomas being the same as the adenocarcinomas described here. The mixed tumors merely consisted of both types of tumors occurring together, a very common occurrence in this series.

As has been mentioned previously, the morphological changes in the K, L, and M groups were the same as in the A to I groups except for the

marked bile duct cell proliferation seen early after m'MeDAB feeding was started. No reference to this bile duct cell proliferation was made by Orr [92], Opie [93], or Edwards and White [94]. This could perhaps be accounted for by the fact that these workers were using the less carcinogenic azo dye DAB. The more potent carcinogen m'MeDAB, however, was used in the first experiment with the groups A to I and in previous experiments in this laboratory, [84] and this bile duct cell proliferation was not seen.

The only reference to this condition found in the literature was in the work of Price, Harman, Miller, and Miller. [95] They found that an increase in the number of bile duct cells began occurring at 18-24 days in the animals fed m'MeDAB and by 26-28 days these had increased in number until they had penetrated close to the central veins of most lobules. By six to eight weeks most of the bile duct cells had disappeared.

In this experiment in the L and M animals, which were pretreated with MCA before being fed m'MeDAB, this proliferation of bile duct cells was first seen after four weeks of m'MeDAB feeding when the first animals were killed. It was found to reach a maximal degree anywhere from 8 to 12 weeks and persist until cirrhosis was marked. In the K animals fed m'MeDAB from the start, the bile duct cell proliferation was noted as early as 2-1/2 weeks in animals that died. It can be seen from this that the bile duct cell proliferation in this experiment reached a maximum later and persisted much longer than in the experiment of Price and associates.

Price and his co-workers postulated that some of these bile duct cells went to form the cholangiofibrosis, but that the majority of them were transformed into parenchymal liver cells. They postulated this to

explain the disappearance of the bile duct cells and the appearance of new parenchymal cells. Further evidence offered to support this was that the new parenchymal cells had a low mitotic index, their mitochondria content was lower than that of the residual parenchymal cells, their nuclear characteristics were more like those of bile duct cells, and they had some tendency to occur around lumina.

This postulate can neither be confirmed nor denied from the results of this experiment. The time relations in this experiment were certainly different than those observed by Price and co-workers, but the morphologic changes described were essentially the same and the impression was obtained that the bile duct cells and the liver parenchymal cells were mutually capable of transformation into the other type. Why the K, L, and M animals showed this change while the A to I animals did not, cannot be explained.

Previous experiments in this laboratory have shown that when 0.0067 per cent MCA is added to a diet containing 0.06 per cent m'MeDAB before the fifth week of feeding liver cancer is completely inhibited. If the MCA is added 6 to 10 weeks after m'MeDAB has been fed, there is some inhibition, but it is not complete. The present experiment showed that feeding MCA for five to nine weeks before giving m'MeDAB or feeding MCA in combination with m'MeDAB for five to nine weeks before giving m'MeDAB by itself also inhibited, but did not completely prevent, liver cancer. The mechanism of this inhibition has never been clarified. This experiment fairly well rules out the possibility that MCA has a "neutralizing" action on m'MeDAB in the intestinal tract, as the two substances were never present together in the intestinal tract. Other possibilities exist which cannot be ruled in or out by this experiment. These include the possibility that MCA might alter the metabolism of the liver in some way so as to make

it more capable of destroying m'MeDAB. If binding of the azo dye by the liver proteins is one of the first steps in carcinogenesis, as is suggested by Miller and his co-workers, MCA might prevent this protein-azo-dye combination and thus prevent the tumors. MCA could conceivably cause a hormonal response in the rat which inhibited tumor formation. The exact mechanism of this inhibition is still obscure and further work will be necessary to elucidate it.

The reasons behind the failure of the liver slice incubation experiment to yield results are unknown. Since this technique has yielded good results in the hands of Kensler and his co-workers [53][54] using liver slices and Mueller and Miller [51] using liver homogenates, it is not likely that the technique is at fault, but rather the application of the technique.

CONCLUSIONS

1. M'MeDAB must be fed to rats a minimum of 10 weeks to produce cancer.

2. An additional 10 weeks, making a total of 20 weeks from the onset of m'MeDAB feeding, is necessary before the cancers begin to appear.

3. There is progressive liver damage including increasing severity of cirrhosis, fatty metamorphosis, bile duct proliferation, bile duct cyst formation, cholangiofibrosis, and nodular hyperplasia before liver cancers appear.

4. Liver cancers are of two types; malignant hepatomas and adenocarcinomas.

5. Pretreatment of rats with MCA for five to nine weeks prior to m'MeDAB feeding inhibits to some extent, but does not prevent liver cancers when m'MeDAB is subsequently fed.

6. MCA fed by itself has no effect on the liver of the rat.

7. Although the mechanism of action of MCA in inhibiting m'MeDAB induced tumors has not been clarified, one possibility, that of "neutralization" in the intestinal tract, has been eliminated.

8. Bile duct cell proliferation was found to occur in three groups (K, L, and M) fed m'MeDAB. This reached a maximum at six to eight weeks and disappeared by the time marked cirrhosis was present.

9. No conclusions could be drawn from the liver slice incubation experiment.

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