Note on the Heat Coagulation of Homogenates from Normal and Precancerous Livers*

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In connection with studies on riboflavin in various fractions of precancerous livers, the observation was made that homogenates of liver tissue from rats fed *m'*-methyl-*p*-dimethylaminoazobenzene failed to coagulate at 100° C., whereas similar homogenates from normal rats coagulated completely in 1 to 5 minutes. Accordingly a study was made of the stability towards heat of homogenates from livers of rats exposed to azo dyes for various lengths of time.

METHODS

Young adult rats of the Sprague-Dawley strain were fed a diet similar to that used in previous studies for the production of hepatic tumors with the azo dyes (10, 13). It had the following composition: casein, 12; salts, 4; corn oil, 5; and glucose monohydrate, 79. Appropriate amounts of the azo dyes, *p*-dimethylaminoazobenzene, aminoazobenzene, or *m'*-methyl-*p*-dimethylaminoazobenzene were dissolved in the oil to yield the concentrations desired (Tables I and II), and the feeding period was continued for intervals up to 16 weeks. The rats were then anesthetized with ether, the abdominal and thoracic cavities were exposed, and the liver perfused with 50 ml. of 0.85 per cent NaCl injected into the vena cava with the portal vein cut. The liver was then removed rapidly and homogenized for 3 minutes in a Waring blendor with 10 ml. of cold distilled water per gram of liver. Such homogenates were stable to centrifugation, to filtration through Whatman 42 filter paper, and to standing for 24 hours at room temperature.

For the coagulation studies 2 ml. aliquots were placed in 5 ml. test tubes and heated in a boiling water bath until visible coagulation occurred. Such coagula could be filtered or centrifuged off leaving a clear or relatively clear supernatant liquid. Homogenates were prepared from normal and precancerous livers and from tumors that had been induced with azo dyes or with methylcholanthrene and their coagulability determined either directly or after dilution with water or with homogenates from normal tissue. Homogenates from normal tissue were also diluted with water (Table I). In a more extensive study, various concentrations of the three azo dyes were fed to a larger group of rats and the animals killed at bi-weekly intervals up to 16 weeks and the coagulability of the livers determined as before.

RESULTS

Homogenates from the livers of rats not fed azo dyes coagulated completely within 1 to 3 minutes at the dilution used (10 cc. H_2O/gm. fresh tissue), whereas homogenates prepared from the livers of rats fed 0.064 per cent *m'*-methyl-*p*-dimethylaminoazobenzene for 6 weeks or more coagulated much more slowly or failed to coagulate after hours of heating (Table I). Homogenates prepared from hepatic tumors induced by the azo dye or from a rat sarcoma induced by the subcutaneous injection of methylcholanthrene showed a similar resistance to coagulation.

The stability of the homogenates to heat was found to depend upon the concentration of the homogenate and on the pH of the preparation. When a homogenate from normal liver coagulating in 1 minute was diluted with an equal part of water, coagulation was not complete until after 4 minutes of heating. A homogenate from a precancerous liver (0.064 per cent *m'*-methyl-*p*-dimethylaminoazobenzene fed for 2 months) that showed partial coagulation after 10 minutes of heating failed to coagulate in 30 minutes after it had been diluted with an equal part of water. On the other hand, an increase in acidity increased the coagulability of the homogenates. Homogenates that had been extremely resistant to coagulation, coagulated immediately under the influence of heat when the pH had been lowered by 0.8 unit. It is doubtful, however, that the difference between the normal and precancerous livers in the coagulability of their homogenates were due primarily to differences in acidity, since the pH of the homogenates from the two types of livers were essentially equal (pH range 7.0 to 7.2).

Mixtures of labile and resistant homogenates usually showed intermediate rates of coagulation

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when exposed to heat. A mixture of equal parts of coagulable and non-coagulable homogenates failed to coagulate, but in the presence of 2 parts of homogenate from normal liver and 1 from precancerous liver, coagulation was complete, although somewhat slower than that due to the normal liver alone (Table I). Homogenates from rat sarcoma agulation was rapid even after 16 weeks of exposure to the compound. On the other hand, in the presence of an equimolecular amount of the highly carcinogenic dye, *m'*-methyl-*p*-dimethylaminoazobenzene, some samples failed to coagulate after only 4 weeks of exposure to the compound. The feeding of *p*-dimethylaminoazobenzene, which

TABLE I: EFFECT OF DIETS CONTAINING *m'*-METHYL-*p*-DIMETHYLAMINOAZOBENZENE ON THE HEAT COAGULATION OF LIVER HOMOGENATES*

Diet	No. of rats	Degree of cirrhosis	Coagulation on heating
0.064% *m'*-Me-DAB†—6 weeks	10	moderate	none after 15 min.
Dye-free diet	10	none	complete—3 min.
A. 0.064% *m'*-Me-DAB—6 weeks	2	moderate	none—2 hrs.
B. Dye-free diet	1	none	complete—3 min.
2 parts A+1 part B			some—15 min.
3.3 parts A+1 part B			none—15 min.
0.5 " A+1 " B			complete—5 min.
1 " B+1 " H_2O			none—15 min.
A. 0.064% *m'*-Me-DAB—2 months	1	severe	some—10 min.
B. Dye-free diet	1	none	complete—1 min.
C. Rat sarcoma due to methylcholanthrene	1		none—1 hr.
1 part A+1 part H_2O			none—30 min.
1 part B+1 part H_2O			complete—4 min.
1 part B+1 part C			none—30 min.
0.064% *m'*-Me-DAB—10 weeks	1	moderate (non-tumorous part)	none—2 hrs.
Hepatic tumor from above			none—2 hrs.

* Homogenates = 10 cc. H_2O/gm. of fresh tissue.
† *m'*-Me-DAB = *m'*-methyl-*p*-dimethylaminoazobenzene.

TABLE II: RELATIVE EFFECT OF CERTAIN AZO DYES ON THE COAGULABILITY OF HOMOGENATES FROM RAT LIVER

Group No.	Diet and Dye Fed	2 weeks Liver	2 weeks Coagulation	4 weeks Liver	4 weeks Coagulation	6 weeks Liver	6 weeks Coagulation	8 weeks Liver	8 weeks Coagulation	12 weeks Liver	12 weeks Coagulation	16 weeks Liver	16 weeks Coagulation
1	No dye—*ad lib.*	Norm.	++++	Norm.	++++	Norm.	++++	Norm.	++++	Norm.	++++	Norm.	++++
2	No dye-restricted*	Norm.	++++	Fatty	+	Norm.	+++			Norm.	++++		
3	.096% *m'*-Me-DAB†	Mild cirrhosis‡	−										
4	.04% *m'*Me-DAB†			Norm.	++++	Norm.	++++			Mild cirrhosis	++		
5	.04% *m'*-Me-DAB† + 10 μg. B_{12}/gm.	Norm.	++++	Norm.	++++	Norm.	++++			Slight cirrhosis	++		
6	.064% *m'*-Me-DAB†	Norm.	++	Mild cirrhosis	−	Norm.	+++						
					−	Cirrhotic	−	Cirrhotic	−	Mod. cirrhosis	−		
7	.06% DAB†	Norm.	++++	Norm.	++++	Near Normal	+++ ++	Norm.	++++	Norm. mild cirrhosis	+++ −	Mild cirrhosis	− −
8	.054% AAB†	Norm.	−	Norm.	++++	Norm.	++++	Norm.	++	Norm.	++++	Norm.	++++

†DAB = *p*-dimethylaminoazobenzene
m'-Me-DAB = *m'*-methyl-*p*-dimethylaminoazobenzene.
AAB = Aminoazobenzene.
*Rats in group 2 received the same amount of food consumed by group 6.
‡Single survivor.

++++ Complete coagulation—1–3 min.
+++ Complete coagulation—3–6 min.
++ Complete coagulation—6–15 min.
+ Slight coagulation after 15 min.
− No coagulation—15 min.

likewise retarded the coagulation of homogenates from normal liver (Table I).

In the series in which azo dyes of various degrees of carcinogenic activity were fed (Table II) livers from the control animals not fed the dye coagulated rapidly on exposure to heat, as did livers from control animals fed only restricted amounts of food. An exception was noted in a single fatty liver, which did not coagulate as rapidly as the normal livers from rats on these diets. In the presence of the noncarcinogenic azo dye, aminoazobenzene, co- is definitely carcinogenic but only about half as active as the *m'*-methyl derivative, likewise resulted in reduced coagulability although a longer period of exposure to the dye was necessary (12 to 16 weeks) before the change became evident (Table II). The loss in coagulability seemed to parallel the gross physical condition of the liver: livers which on gross inspection showed a moderate number of cirrhotic nodules usually formed homogenates that failed to coagulate on exposure to heat; severely cirrhotic livers always failed to coagulate, whereas

homogenates from livers containing only a few cirrhotic nodules sometimes coagulated, although more slowly than normal.

DISCUSSION

A number of factors are probably responsible for the observed difference in coagulability of the homogenates of normal and precancerous liver. Others have reported that the addition of certain sugars (1, 3, 8) or fatty acids (2, 4-6, 9) may protect albumins against denaturation or coagulation with heat. Carter and Greenstein (7) observed that aqueous extracts of fresh liver from normal rats failed to coagulate when heated, but that they became heat-coagulable on standing at room temperature. The addition of thymus nucleic acid to such coagulable extracts prevented coagulation. Thymus nucleic acid also stabilized solutions of egg albumin which remained water clear for many hours at 98° C. when 1 mgm. of nucleate was present per 600 mgm. of protein. No experiments were reported on extracts from tumors or from precancerous livers.

That nucleic acid may have been one of the factors delaying coagulation in our present experiments is suggested by the observation that hepatic tumors due to azo dyes contain more nucleic acid than normal liver (12, 14). Lipids may also have been involved. Williams and associates (15) have reported that *p*-dimethylaminoazobenzene alters the distribution of lipids in the nucleus of the liver cell, with an increase in cholesterol ester and a significant decrease in phospholipid. But the most important factor is probably the concentration of coagulable protein present in the homogenate. The rate of coagulation of extracts from normal liver was shown to diminish as the concentration of total liver substance decreased. Morrione (11) has reported that the concentration of collagen is doubled in cirrhosis due to *p*-dimethylaminoazobenzene. The presence of the extra collagen would tend to delay coagulation of the entire extract by replacing, and therefore lowering the concentration of coagulable protein, and it might also delay coagulation by a more direct mechanism. Furthermore, the coagulable proteins in normal liver may have themselves undergone subtle changes under the influence of the azo dye. But whatever the mechanism involved, a simple measurement of coagulability may prove of value as a rapid index of the degree of liver damage that has taken place under any particular experimental regimen. This is suggested by the fact that delayed coagulation became evident about the same time that cirrhotic nodules appeared.

SUMMARY

1. Homogenates of livers from rats fed diets containing *m'*-methyl-*p*-dimethylaminoazobenzene for 6 weeks or longer failed to coagulate when heated in a boiling water bath. Normal liver homogenates coagulated completely after being heated for only a few minutes.

2. Resistance to coagulation paralleled the number of cirrhotic nodules. When 0.064 per cent *m'*-methyl-*p*-dimethylaminoazobenzene was fed, only 4 to 6 weeks were required before resistance to coagulation became marked, whereas 12 to 16 weeks were required before comparable resistance developed under the influence of 0.06 per cent *p*-dimethylaminoazobenzene. Rats fed the non-carcinogenic *p*-aminoazobenzene for 4 months were normal in appearance, and the homogenates prepared therefrom coagulated like those from normal livers.

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