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Genotoxicity, Carcinogenicity, and Mode of Action of the Fried Food Mutagen 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ)

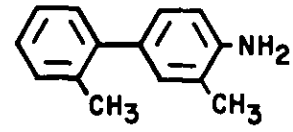
by John H. Weisburger,* William S. Barnes,*† Charles A. Lovelette,*† Charles Tong,* Takuji Tanaka,*§ and Gary M. Williams*

Because mutagens typified by 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) observed in cooked foods are widely consumed, detailed studies of their biochemical and biological properties including carcinogenicity are most important. IQ induces unscheduled DNA synthesis in liver cells, which when taken together with its powerful mutagenicity in the *Salmonella typhimurium* test system, predicts carcinogenicity. In female Sprague-Dawley rats, IQ did exhibit potent carcinogenicity for the mammary gland, the ear duct, and to a lesser extent, pancreas and bladder. Data from Japanese laboratories indicate carcinogenicity also to the intestinal tract. Thus, one of the mutagens formed during cooking is a versatile carcinogen that because of extensive human intake requires urgent exploration for specific human cancer risk.

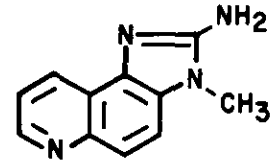
Introduction

The discovery by Sugimura et al. (*1,2*) that the surfaces of fried meat or fish contain powerful mutagens opened an important new area in food toxicology and nutritional carcinogenesis. Isolation of these mutagens identified them as heterocyclic aromatic compounds with an exocyclic amino group and often an *o*-methyl group (*3*). This newly discovered class of mutagens may be the as yet unknown carcinogens for the nutritionally linked cancers (*4*). Thus, it is important to acquire detailed information on their biological properties and their mode of action.

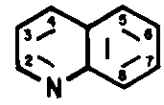
Inspection of the structures of the food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), the synthetic chemical 3,2′-dimethyl-4-aminobiphenyl (DMAB) (a carcinogen for the colon, breast, and prostate), and quinoline shows that IQ contains both the quinoline ring system and the primary amine with an *o*-methyl substituent, which characterizes DMAB. Since IQ is both a mutagen and a carcinogen, it is important to establish whether this compound is activated by oxidation of the amino group or whether ring epoxidation, such as may occur with quinoline, is also relevant.



3,2′-dimethyl-4-aminobiphenyl



2-amino-3-methylimidazo[4,5-f]quinoline



quinoline

Experiments presented here demonstrate that IQ, one of the mutagens found in fried meat, is not only a powerful mutagen in the Ames *Salmonella typhimurium* test system but can also induce unscheduled DNA synthesis (UDS) in the primary culture rat hepatocyte (HPC) test of Williams. Also, although quinoline can act as an initiator in carcinogenicity tests on the skin of

*Naylor Dana Institute, American Health Foundation, Valhalla, NY 10595-1599.

†Present address: Department of Biology, Clarion University of Pennsylvania, Clarion, PA 16214.

‡Present address: Department of Chemistry, Norwich University, Northfield, VT 05663.

§Visiting pathologist; permanent address: Gifu University School of Medicine, Gifu City 500, Japan.

mice promoted with phorbol ester, IQ fails to act as an initiating agent, and the homocyclic analog 3,2'-dimethyl-4-aminobiphenyl likewise appears negative as an initiator. Furthermore, in tests of the mutagenicity of analogs of IQ, only the chemicals with an exocyclic amino group had powerful activity. It seems likely that mutagenicity and other reactions involving attack on DNA require the *N*-oxidation of exocyclic amino groups. Ring epoxidation is probably not a likely activation step.

Any compound active as a mutagen in the Ames test and capable of inducing DNA repair in the Williams HPC test is likely to be a carcinogen (*5*). We now find this prediction verified. IQ is a potent carcinogen for the rat mammary gland in comparison to an established positive control carcinogen for this organ, 4-aminobiphenyl (4-AB). Also, IQ induced Zymbal's gland tumors, and to a lesser extent, neoplastic nodules, carcinomas and hemangioendotheliomas in the liver, pancreatic neoplasms, and renal pelvis and urinary bladder neoplasms. Furthermore, preneoplastic lesions were seen in the liver, pancreas, and adrenal cortex. Thus, IQ is a multipotent carcinogen for the rat by affecting a variety of target organs. These data together with those obtained by Ohgaki et al. (*6,7*) and Takayama et al. (*8,9*) suggest that specific modes of cooking lead to carcinogens in mice and rats of several strains that exhibit an organotropism simulating that seen in the nutritionally linked cancers in the Western World.

Materials and Methods

Chemicals and Media

Gold-label (99% pure) quinoline was purchased from Aldrich Chemical Co. (Milwaukee, WI) and redistilled prior to use. Purity established by gas chromatography was greater than 98%. DMAB was purchased as the hydrochloride salt from Ash-Stevens (Detroit, MI). The free base was obtained by extraction from a saturated solution of sodium bicarbonate into diethyl ether. Extracted DMAB was eluted through a Clin Elut disposable column (Fisher Scientific, Pittsburgh, PA), and solvent was removed by rotary evaporation. No impurities were detectable by HPLC; the correct mass spectrum completed structural identification. IQ was purchased from Toronto Research Chemicals, Toronto, Canada (Dr. David Dime). No impurities were detectable by HPLC; the compound had the correct mass spectrum (*10*).

4-AB was purchased from Aldrich Chemicals (Milwaukee, WI). For the carcinogenicity tests, IQ and 4-AB were converted to the hydrochloride salt by using an excess of gaseous HCl in methanol. The product was then filtered, washed with cold ethanol and ether, and stored at 4°C until used.

12-O-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Consolidated Midland (Brewster, NY).

Williams' Medium E (WME) and calf serum were obtained from Flow Laboratories (Rockville, MD). [^{3}H]TdR, 60–80 Ci/mmole, was purchased from New England Nuclear (Boston, MA). NTB, D19 developer, and fixer were from Eastman Kodak (Rochester, NY). Media for bacterial mutagenicity assays were from Difco Laboratories (Detroit, MI). NADP and glucose-6-phosphate were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Imidazoquinoline Derivatives

2-Amino-1H[3H]imadazo[4,5-f]quinoline was prepared in the following fashion. 5-Amino-6-nitroquinoline (Aldrich) was reduced with tin(II)chloride and concentrated hydrochloric acid followed by appropriate workup to 5,6-diaminoquinoline (*11,13*), which was treated with cyanogen bromide in refluxing methanol, bringing about final closure of the imidazole ring. 5,6-Diaminoquinoline served as the key precursor in the preparation of 1H[3H]imidazo[4,5-f]quinoline and 2-methyl-1H[3H]imidazo[4,5-f]quinoline where final ring closure was accomplished by reflux in the appropriate acid. 3-Methyl-imidazo[4,5-f]quinoline was prepared by a slight modification of the method of Myers and Zeleznick (*14*), which involved lyophilization of 1H[3H]imidazo[4,5-f]quinoline and tetramethylammonium hydroxide (10% aqueous) followed by sublimation of the final product at 200°C and and 0.5 torr. Proton NMR spectral data indicate that N-3 alkylation predominates over N-1 substitution (*15*). The purity of all compounds described above was established by TLC (silica, methylene chloride, ethyl acetate, 1:1 V/V, and hexane, methyl alcohol 9:1 V/V). Final purification was accomplished by preparative layer chromatography techniques, if required.

Mutagenicity Assay

Salmonella typhimurium tester strains TA 98 and TA 100 were obtained from B. N. Ames (*16,17*). The S9 fraction of liver was prepared from male Sprague-Dawley rats induced with Aroclor 1254 (*17*). Each assay involved 100 μL of an overnight culture of bacteria added to 2 mL of soft agar containing histidine and biotin, and 0.5 mL of S9 mix containing 50 μL of S9 fraction (40 mg protein/mL). The test compounds were dissolved with 100 μL of DMSO. The mixture was vortexed and overlaid onto Vogel-Bonner minimal agar plates, then incubated for 48 hr at 37°C and counted on an Artek Model 880 colony counter. Controls included vehicle control, positive control (2-aminoanthracene for both TA 98 and TA 100), and sterility controls containing soft agar + test agent, soft agar + S9 mix, and soft agar alone. Spontaneous revertant frequencies in this series of experiments were 40 ± 9/plate with S9 for TA 98, 140 ± 15/plate with S9 for TA 100. Revertant/nmole of compound and revertants/μg of compound were calculated by linear regression analysis of the data, without subtracting spontaneous revertant frequencies.

Assay for UDS in Rat HPC

Hepatocyte Preparation. The livers of adult rats were perfused *in situ* with collagenase as described (*18*).

Preparations with a cell viability greater than 85% were used. About 5×10^5 viable cells were plated in a Linbro dish (Flow Laboratories, Rockville, MD), with each well containing a 25-mm^2 round Thermox plastic coverslip. The cells were allowed to attach for 2 hr in WME containing 10% calf serum in a 5% CO_2 incubator, then washed and refed with WME.

HPC/DNA Repair Test. Immediately after the 2-hr attachment period, hepatocytes were simultaneously exposed to [^{3}H]TdR (10 μCi/mL) and the test compound dissolved in DMSO. The maximum concentration of DMSO was 1%. After 18 hr, the coverslips were processed as previously described (*18*). Air dried coverslips, mounted on glass slides, were dipped in NTB emulsion, then developed and stained after 10 days (*18*). Nuclear and background grain counts were determined with an Artek Model 880 counter using the area mode. Three cytoplasmic counts were obtained for each nucleus. Net nuclear counts were calculated by subtracting the highest cytoplasmic count from the nuclear count for each cell. The data are expressed as the mean net nuclear count for three slides ± the standard deviation. A net nuclear count of 5 grains or more represents a positive response (*18*).

Bioassay for Initiating Activity on Skin of Sencar Mice

Female mice, aged 49–51 days, were purchased from Sprague-Dawley (Indianapolis, IN). A solution of 6 mmole of chemical was applied to the shaved backs of mice in 0.1 mL of DMSO three times per week for 2 weeks. Ten days later the promoting regimen was applied as 2.1 mg TPA in 0.1 mL acetone twice a week for 20 weeks. Twenty animals were treated with vehicle only, 20 with quinoline, and 30 each with IQ and DMAB. The mice were examined weekly and papillomas were scored when they reached a size of 7 mm in diameter. Representative samples of gross tumors were submitted for pathological examination.

Carcinogen Bioassay in Female Rats

Weanling female Sprague-Dawley rats (Sprague Dawley, Madison, WI) were maintained in quarantine for 2 weeks while routine health tests were performed. At 6 weeks of age, a total of 100 rats found suitable for bioassay were transferred to holding rooms of the Research Animal Facility, randomized into groups of 32 each experimental rats, 27 vehicle control rats, and 9 untreated control rats, and were kept three to a polycarbonate cage with hardwood bedding. The rooms were maintained at 23 ± 2°C and 40–60% humidity with 8 to 10 air changes per hour and with a 12-hr light/dark cycle. Drinking water (tap) was provided by an automatic distribution system.

During the quarantine period, all rats were fed the standard NIH-07 diet *ad libitum*. At 6 weeks of age, beginning with the chemical treatment period, all rats, including controls, were switched to a diet consisting of standard NIH-07 to which 15% corn oil was added for a total fat content of 20% by weight.

The hydrochloride salts of IQ and 4-AB were dissolved in a solution of 5% Emulphor (EL 620, GAF Corp., New York) in distilled water at a concentration such that 0.4 mmole/kg body weight of IQ or 0.22 mmole/kg of 4-AB were present in 0.25 mL solutions. These solutions were administered by gavage on the following regimens. The first doses were given to rats when they were 6 weeks old and continued three times per week for weeks 1–4, at a time when the female Sprague-Dawley rat is maximally sensitive to the induction of mammary tumors. However, because of beginning toxicity, the three times per week treatment for 4-AB was administered only from weeks 1 to 3. For IQ, the gavage was continued at two times per week from weeks 5 to 8, and for 4-AB, this protocol was used from weeks 4 to 8. For the next 23 weeks, the dosage was administered once a week. After that time, all of the animals continued without further chemical treatment on the control diet. One group of rats was given the vehicle control, 0.25 mL 5% Emulphor solution in distilled water on the same schedule as the IQ group. An additional group of nine rats served as untreated controls.

Beginning 8 weeks after the first gavage treatment, the animals were carefully palpated to detect mammary tumors (*19*). All rats were killed 52 weeks after the first dose. They were necropsied, any abnormalities were noted, including the multiplicity of tumors. The tissues were fixed in 10% buffered formalin and sections were routinely prepared and stained for microscopic diagnosis. Histologically, mammary tumors were diagnosed according to the classification of Young and Hallows (*20*). Liver neoplasms were diagnosed as neoplastic nodules, hepatocellular carcinomas, or hemangioendotheliomas according to the criteria described by Stewart et al. (*21*).

Results

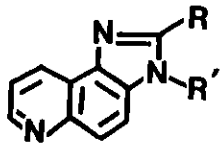
Mutagenicity Tests of IQ Analogs

In comparison to the powerful mutagenic effect of IQ in the frameshift-sensitive *Salmonella typhimurium* TA 98 strain and the lower degree of activity in the base-pairing indicator of the *Salmonella typhimurium* TA 100 strain, the chemical without a methyl substitution had much lower activity in the TA 98 organism and none at all in TA 100 (Table 1) (*22*). The compound without the exocyclic amino group but with an *N*-methyl substitution had a small but definite activity both in the base-pairing and frameshift sensitive tester strains. The other compounds had no activity.

Assay for Unscheduled DNA Synthesis (UDS)

A dose level of 1.25×10^{-3} M of IQ is toxic to the liver cells (Table 2). Concentrations of 1.25×10^{-5} M and 1.25×10^{-6} M elicited powerful DNA repair, to a

Table 1. Mutagenicity of IQ and its derivatives in *Salmonella typhimurium*.[a]



			TA 98		TA 100	
			Revertants/plate[b]	Revertants/nmole[c]	Revertants/plate	Revertants/nmole[c]
I (IQ)	R = NH_2;	R′ = CH_3	680/0.003	55,000	800/0.10	1,500
II	R = NH_2;	R′ = H	440/0.10	790	0	0
III	R = H;	R′ = CH_3	430/1000	0.08	390/250	0.30
IV	R = CH_3;	R′ = H	0	0	0	0
V	R = H;	R′ = H	0	0	0	0

[a] S9 mix was present. From Barnes et al. (*22*).
[b] Revertants per plate/mass compound per plate (μg).
[c] Calculated by linear regression analysis from the linear portion of the dose-response curve.

greater extent than an equivalent level of DMAB. Even a concentration of 1.25×10^{-7} M was positive. Quinoline was weakly positive and exhibited 15 grains/nucleus at the fairly high concentration of 1.0×10^{-3} M.

Initiating Activity on the Skin of SENCAR Mice

In a test series conducted by LaVoie et al. (*23*) in this Institute, quinoline was applied in acetone solution to the skin of SENCAR mice. After 20 weeks, 13 of 20 animals (65%) had papillomas, and there were a total of 19 tumors. The current experiment utilized similar protocols and was performed at the same time with the same shipment of mice. However, because of the relative insolubility of IQ in acetone, this test was performed with all chemicals applied as solutions in DMSO. Under these conditions, quinoline initiation yielded skin tumors in 70% of animals, and a total of 27 papillomas in 20 animals (Fig. 1) (*22*). Thus, the DMSO solution of quinoline gave a higher tumor yield compared to the acetone solution. On the other hand, IQ and, interestingly, DMAB did not act as initiators on mouse skin in this sensitive system, both as regards the number of tumor-bearing animals or the tumor multiplicity.

Carcinogen Bioassay in Female Sprague-Dawley Rats

At the beginning of the test, the average weight of all rats was 126 g. At week 10, group 1 (untreated) weighed an average of 270 g; group 2 (vehicle), 274 g; group 3 (IQ), 256 g; and group 4 (4-AB), 247 g. At week 31, at the end of the treatment, the weights were 371, 389, 343, and 347 g, respectively. Thus, the groups given IQ or 4-AB gained less weight by about 10% compared to controls, indicating that the dosage administered was consistent with proper toxicologic practice. In the early weeks of the tests, the rats given 4-AB, but not those on IQ, turned blue for some hours after each gavage, suggesting an effect on the hematopoietic system. This observation was made even though the dose rate for 4-AB (0.22 mmole/kg) was about one-half that of IQ (0.4 mmole/kg). Survival in all groups was good, since 97/100 rats were alive at 6 months. However, three to five rats in the groups receiving IQ or 4-AB died or had to be killed each subsequent month because of the occurrence of cancers, especially in the mammary gland or ear duct. In all groups, 78 of the starting 100 rats were alive when the test was terminated at 52 weeks.

Table 2. Unscheduled DNA synthesis in primary explant cultures of hepatocytes from F344 male rats exposed to IQ and DMAB.[a]

		DNA repair	
Chemical	Concentration, M	Grains/nucleus	% positive nuclei[b]
IQ	1.25×10^{-3}	toxic	—
	1.25×10^{-4}	9.7 ± 3	48
	1.25×10^{-5}	130 ± 21	98
	1.25×10^{-6}	150 ± 18	97
	1.25×10^{-7}	12 ± 8	37
DMAB	1.0×10^{-6}	85 ± 27	

[a] From Barnes et al. (*22*).
[b] Positive response = > 5 grains/nucleus.

Neoplasms in the Mammary Gland. The major finding in this bioassay was the induction of cancer in the mammary gland. With IQ, the first palpable tumor was noted during the 12th experimental week and with the positive control carcinogen 4-AB during week 16. Progressively more mammary tumors were palpated during the test. No regression of palpable tumors was noted. At the end of the 5-week study period, 14 of the 32 rats given IQ exhibited 21 mammary carcinomas and one a hemangioendothelioma (Table 3) (*24*). In the group of 32 rats given 4-AB, 18 had 32 mammary carcinomas, 2 had fibroadenomas, and 1 a fibrosarcoma. In the 27 control rats intubated with vehicle, 2 had three mammary fibroadenomas. None of the nine untreated controls had palpable or microscopic mammary gland tumors.

Zymbal's Gland Neoplasms. Eleven of the thirty-two rats given IQ had 12 keratinizing epidermoid carcinomas in the ear duct (Table 3). The earliest growth appeared at 40 weeks. One animal had bilateral tumors. The right ear duct was involved in 6/10 tumors. This

Table 3. Incidence of tumors in female Sprague-Dawley rats treated with IQ or 4-AB.[a]

Group	Treatment	No. of rats	No. of rats with tumors	No. of animals									
				Mammary tumors				Liver tumors[b]				Ear duct tumors	Other tumors
				Total	Carcinoma	Fibroadenoma	Others	Total	NN	HC	Others		
1	Control	9	0	0	0	0	0	0	0	0	0	0	0
2	Vehicle control	27	2	2 (3)	0	2 (3)	0	0	0	0	0	0	0
3	IQ	32	23*	14* (22)	14* (21)	0	1[c] (1)	6* (9)	3 (5)	2 (2)	2[c] (2)	11* (12)	5[d] (6)
4	4-AB	32	20*	19* (35)	18* (32)	2 (2)	1[e] (1)	0	0	0	0	0	0

[a] Numbers in parentheses are the total number of tumors. From Tanaka et al. (*24*).
[b] NN: neoplastic nodule, HC: hepatocellular carcinoma.
[c] Hemangioendothelioma.
[d] Granulocytic leukemia, two rats: pancreatic acinar cell adenoma, one rat: islet cell adenoma, one rat: transitional cell papillomas of pelvis and urinary bladder, one rat.
[e] Fibrosarcoma.
* Significantly different from Group 2 by Fisher's exact probability test ($p < 0.05$).

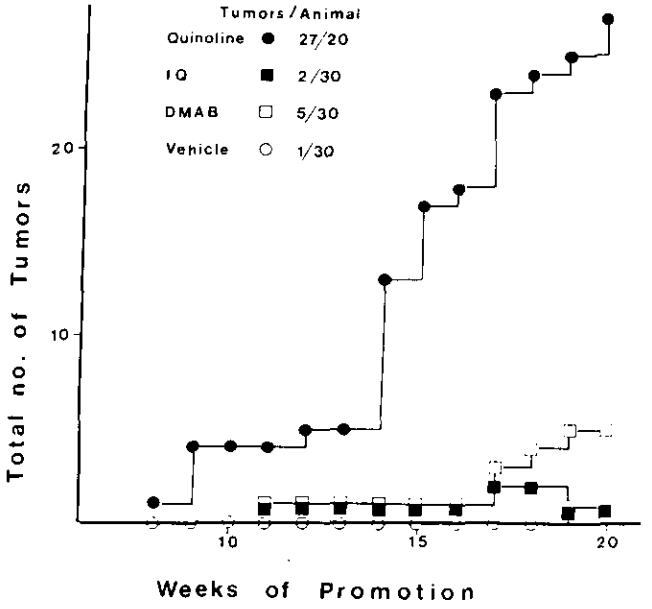


FIGURE 1. Weekly incidence of total skin tumors on animals treated with quinoline, IQ, DMAB, and vehicle (DMSO). The total number of tumors per animal was: quinoline 17/20, IQ 2/30, DMAB 5/30, and vehicle 1/30. The number of tumor bearing animals was: quinoline 70%, IQ 7%, DMAB 17%, and vehicle 3% (*22*).

lesion was absent in rats given 4-AB or either of the two control groups.

Miscellaneous Preneoplastic or Neoplastic Lesions. In the IQ group, 17/32 rats had altered liver cell foci (Table 4), three had five neoplastic nodules, two had hepatocellular carcinomas, and two more had hemangioendotheliomas in the liver (Table 3). With 4-AB, 5/32 had altered liver cell foci but no liver neoplasms (Tables 3 and 4). The controls had normal livers.

In the IQ group, 19/32 had atypical hyperplastic acinar cell lesions in the pancreas, one had an acinar cell adenoma, and one had an islet cell adenoma of the pancreas (Tables 3 and 4). With 4-AB, only 7/32 had atypical hyperplastic acinar cell lesions but failed to show neoplasia in the pancreas.

Table 4. Incidence of preneoplastic lesions in female Sprague-Dawley rats.[a]

	No. of rats with preneoplastic lesions			
	Group 1, control	Group 2, vehicle control	Group 3, IQ	Group 4, 4-AB
Liver: altered liver cell foci	0	0	17* (53)	5* (16)
Pancreas: atypical hyperplastic acinar cell lesions	0	0	19* (59)	7* (22)
Adrenal cortex: altered proliferative foci	0	0	5* (16)	2 (6)

[a] From Tanaka et al. (*24*). Numbers in parentheses are % of lesion-bearing rats.
* Significantly different from Group 2 by Fisher's exact probability test ($p < 0.05$).

With IQ, five altered proliferative foci in the adrenal cortex were seen, and there were two in the group given 4-AB (Table 4). In addition, in the group of rats given IQ, two had leukemia and one had a papilloma of the pelvis and urinary bladder (Table 3). None of the control or vehicle control rats had abnormalities in these organs.

Discussion

IQ, one of the important new mutagens found in fried, broiled, or cooked meat or fish, was a good inducer of unscheduled DNA synthesis in liver cells. We have observed that a chemical that is reliably active in both the bacterial mutagenicity screen of Ames and the mammalian DNA repair system of Williams is most likely a genotoxic carcinogen. The data presented indicate IQ

to be quite active in both tests classifying it as a genotoxic carcinogen (*5,25*).

On the other hand, the results of the assay for initiating potential on mouse skin, in contrast to the positive control quinoline, show that IQ and the related homocyclic aromatic amine DMAB cannot act as initiators in the skin of the sensitive SENCAR mouse strain, probably because the reactive electrophilic metabolites do not reach the skin in sufficient amount under the conditions of this test. In contrast, chronic feeding of the carcinogenic aromatic amine 2-acetylaminofluorene (AAF) revealed initiating potential on mouse skin when followed by promotion by croton oil (*26*), or by subcutaneous injection of carrageenan in rats (*27*). Interestingly, a higher incidence of skin tumors was obtained, upon promotion with croton oil in mice fed with AAF than in mice fed with its *N*-hydroxy derivative, the proximate carcinogen (*28*). The *N*-hydroxy derivative, normally considered more reactive, was less active (*28*), and thus, the initiating action might stem from ring epoxidation rather than *N*-oxidation. With quinoline itself, LaVoie et al. (*29*) noted that the 5,6-epoxide is a possible carcinogenic form. In IQ, the imidazole ring occupies the 5,6-region, which may account for the failure of IQ to act as an initiator.

The combined data obtained, therefore, suggest that IQ and related heterocyclic mutagens and carcinogens most likely are converted to proximate and ultimate carcinogenic forms by *N*-oxidation on the exocyclic amino group. The results of the mutagenicity test of typical chemicals in this series point in the same direction. Furthermore, the metabolism experiments so far reported indicate that *N*-hydroxylation is an essential activation reaction (*30*).

In agreement with the positive results from the battery of *in vitro* tests described herein and elsewhere (*3,31–35*), IQ has been found to be a multipotential, powerful carcinogen in the rat. In this test, the chemicals were administered by gavage to obtain quantitative information of dosage and also to use the expensive chemical IQ most economically. The total dosage administered per rat was 4.4 mmole (871 mg) of IQ and 2.4 mmole (400 mg) of 4-AB, the positive control carcinogen. The overall duration of the test was 1 year. Thus, with intermittent, limited administration of the test chemical, a high yield of mammary gland cancer was induced. In addition, other neoplasms were found, including those in the ear duct, and to a lesser extent, in the liver, pancreas, and urinary tract. Compared to the positive control, the human carcinogen 4-AB, IQ can be considered to have approximately the same potency allowing for the 2-fold lower level of 4-AB administered. Nonetheless, IQ demonstrated a greater versatility, and yet specificity, by inducing cancer mainly in the mammary gland and ear duct. The induction of mammary gland neoplasms may have been potentiated by dietary fat as has been noted with other mammary carcinogens, especially the closely related homocyclic 3,2′-dimethyl-4-aminobiphenyl (*36*). In addition, Takayama et al. (*7,8*) observed the induction of cancer in the intestinal tract, including small and large intestines as well as lesions in the pancreas and ear duct, when 300 ppm IQ was fed in the diet to Fischer strain rats.

The data available thus far suggest that IQ, one of the mutagens formed during the cooking of foods is a potent carcinogen in mice and in rats of two strains. Because IQ affected those target organs, intestional tract, breast, and pancreas, among others, that represent the main nutritionally linked cancers in the Western World, it is important to extend these findings and determine whether the mutagens found in fried food are the genotoxic carcinogens associated with major forms of human cancer (*37*).

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