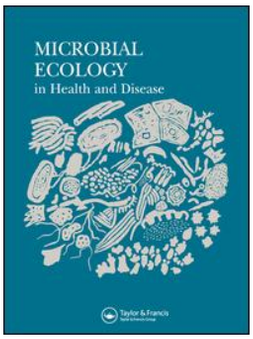

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Bacterial Metabolism in Humans of the Carcinogen IQ to the Direct Acting Mutagen Hydroxy-IQ

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7-hydroxy-IQ is the major product of the bacterial metabolism of IQ, a potent dietary carcinogen. Yet, unlike IQ, hydroxy-IQ is directly active in the salmonella/microsomal mutagenicity assay. Two subjects consumed a meal of fried meats containing IQ but no detectable hydroxy-IQ. Hydroxy-IQ was isolated from the subjects' faeces collected within 30 h following the fried meat meal; it was absent from the subjects' faeces before and after the meal. This is the first evidence that hydroxy-IQ is formed in humans. Subsequent concern is therefore raised as to its role in the aetiology of colon cancer. From the faeces of one of the subjects a strain was isolated which conformed to the description of a *Eubacterium* sp. and was capable of producing hydroxy-IQ.

KEY WORDS—2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline; IQ; 2-amino-3,6-3dihydro-3-methyl-7*H*-imidazo[4,5-*f*]-quinoline-7-one; Hydroxy-IQ; Intestinal flora; Carcinogens; Faecal Mutagens; *Clostridium*; *Eubacterium moniliforme*.

INTRODUCTION

Pyrolysis carcinogens are formed when proteinaceous foods, especially meats, are cooked at high temperatures[9,25] and they have been shown to be responsible for the rise in faecal mutagenicity upon ingestion of fried beef.[14,15] Consequently, we believe it is important to understand the fate of dietary pyrolysis carcinogens once they are ingested. One group of pyrolysis carcinogens, the 'IQ-class' (2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; IQ, MeIQ and MeIQx respectively), is common in fried beef[12,13] and includes some of the most potent genotoxins found in faeces. Upon activation with liver microsomes, IQ is highly mutagenic in bacterial test systems.[10] IQ is also genotoxic in mammalian cells[19] and induces tumours in several organ sites in rats.[21,27] As a result, the occurrence of the 'IQ-class' of carcinogens in human diets, especially so-called 'western diets' rich in cooked meats, has generated much concern about the role of these compounds in human carcinogenesis.

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We recently showed that IQ is hydroxylated *in vitro* to 2-amino-3,6-dihydro-3-methyl-7*H*-imidazo[4,5-*f*]quinoline-7-one (hydroxy-IQ). This occurs in human faeces and pure cultures of *Eubacterium* and *Clostridium* spp.[4,5,7] We also showed that unlike IQ, hydroxy-IQ is a direct-acting mutagen in the salmonella/microsome mutagenicity test;[7] we are currently testing it for carcinogenicity in rodents. In this report we describe the isolation of hydroxy-IQ from human faeces of two healthy adult human volunteers following their consumption of a large meal of fried meats. We also describe the selective methods required for isolation of bacteria from faeces which are capable of hydroxylating IQ.

MATERIALS AND METHODS

Reagents

Synthetic IQ and hydroxy-IQ were prepared as described previously.[4,5] Stock solutions of IQ or hydroxy-IQ in DMSO (1 mg/ml) were filtered (0·2 μm) and stored under nitrogen at 4°C. Stock solutions of MeIQ and MeIQx were prepared as above.

Bacteriological media and supplements

Pre-reduced and anaerobically sterilised brain heart infusion (BHI) broth (9 ml/tube) and agar

Table 1. Timecourse of the fried meat consumption

Dietary scheme	Normal eating (48 h)	Fasting (18 h)	Meat (6 h)	Fasting (18 h)	Normal eating (12 h)	(48 h)
Subject A Pools*	3 samples (375 g)			2 samples (295 g)		2 samples (280 g)
Subject B Pools	4 samples (405 g)			2 samples (325 g)		3 samples (355 g)
	'pre-meat'			'meat'		'post-meat'

*The 'pre-meat' pools of faecal samples were samples collected 48 h prior to and during an 18 h period of fasting. The 'meat' pools were any samples collected over the 30 h after beginning consumption of the fried meats. The 'post-meat' pools were samples collected 30–78 h after beginning consumption of the fried meats.

were made following the description in the Anaerobe Laboratory Manual.[16] Because resazurin is a coplanar tricyclic molecule, and as such would be extractable with blue cotton, it was omitted from the formulations. Stock solutions of neomycin and kanamycin were prepared in BHI broth and filter sterilised (0·22 μm). Antibiotics were added, aseptically and anaerobically, to solid or liquid media to give final concentrations of 100 μg neomycin/ml and 75 μg kanamycin/ml.[29] Tween-80 was added to all broths according to the method of Holdeman *et al.*,[16] to give a final concentration of 0·02 per cent vol/vol. Broth media was incubated at 37°C overnight prior to use; agar plates were similarly preincubated in an anaerobic atmosphere (90 per cent H_2, 10 per cent CO_2).

Diet

Two of the authors (non-smokers), whose faecal flora produced hydroxy-IQ *in vitro*,[7] fasted for 18 h, then consumed a 'meal' of fried ground beef, ham and sausage over a 6 h period. They then fasted for another 18 h before resuming their normal diets (Table 1). Faecal samples were collected throughout this period.

Portions consumed by each subject during the 6 h period consisted of eight 100 g hamburgers (15 per cent fat), four 50 g sugar-cured ham slices and six 50 g sausage patties. All weights were determined before cooking. Meats were cooked in accordance with conditions for maximising production of the pyrolysis carcinogens.[20,22–24] The meats were fried in an electric frying pan in which the heating element was sealed in oil; this acted as a heat sink which allowed even distribution of heat over the entire cooking surface. Each portion was fried at 225°C for 5 min/side. Heat transfer from the bottom of the frying pan to the meat patties was optimised by not draining the fat from the pan until all the respective patties were fried. Equal portions of fried meats to those consumed by each subject were frozen at −70°C for processing as controls. Water and diet soft drinks were available *ad libitum*. No other foods were eaten between the two fasting periods. We used faeces passed 48 h before and after the meal as negative controls.

Faeces

Faecal samples were passed directly into sterile zip-lock bags which were flushed with nitrogen and frozen at −70°C until processed. The faeces were pooled, thawed and thoroughly mixed at room temperature in a fresh bag under nitrogen.

Positive controls for our isolation studies consisted of 10 g faeces suspended in 10 ml sterile anaerobic diluents[16] which were supplemented with radiolabelled IQ and hydroxy-IQ. The tubes were stoppered under anaerobic CO_2 and immediately frozen at −70°C until processed. IQ and hydroxy-IQ were extracted from diluted faeces with 10 mg blue cotton per ml (Mutasorb, Pierce Chemical Company) as previously described.[7]

Food and Faecal Homogenates

After the three collection periods (Table 1) the faecal samples from each subject were thawed, mixed in a 2 litre flask and diluted with four volumes (wt/vol) of double deionised water. Each of the three faecal pools were homogenised and centrifuged at 13 000 *g* for 1 h at 20°C. The supernatants were decanted into 2 litre flasks. The fried meat controls were thawed, macerated, blended with four volumes of double deionised water, centrifuged and decanted

into 2 litre flasks. Blue cotton was added to the supernatants of each homogenate (2·5 g/250 ml) and the mixtures shaken for 30 min at room temperature.[18] The blue cotton from each homogenate was rinsed with double deionised water, thoroughly dried using a hair dryer and extracted with 100 ml methanol/ammonia (99:1). The homogenates were extracted a second time with blue cotton (as above) and the two blue cotton extracts were pooled and flash evaporated at 60°C. The residues were dissolved in 250 µl methanol, diluted with 250 µl chloroform, filtered (0·5 µm), and stored at 4°C until analysed by high performance liquid chromatography (HPLC).

HPLC

We isolated IQ and hydroxy-IQ from the blue cotton extracts of faeces of each subject using two HPLC systems. The first was normal phase (semi-preparative) silica using a radial compression module (RCM) silica cartridge (Millipore, Waters Div.) with chloroform/isopropanol (60:40). To determine where IQ and hydroxy-IQ would elute, blue cotton extracts of 'normal' faeces were spiked with 2 µg/ml synthetic IQ and hydroxy-IQ and run in this semi-preparative system. After determining the elution period for each compound, we 'rinsed' the system thoroughly by injecting 200 µl of solvent through the system in three consecutive runs to remove any traces of either compound. Immediately following this rinse period, the 0·5 ml blue cotton extracts of 'meat' faeces, in which IQ and hydroxy-IQ were minor components, were run through the system as five 100 µl injections. We pooled the fractions containing each compound and evaporated them to 0·5 ml. These two samples, in which IQ or hydroxy-IQ were now major components were then re-run through the same system for further purification. The peaks for IQ and hydroxy-IQ were collected, pooled, evaporated and re-suspended in 0·5 ml methanol.

We next ran the samples through the second HPLC system, reverse phase C_{18}, using an RCM Nova-Pak C_{18} cartridge with water/methanol/ammonia (75:25:1). Fractions containing the purified compounds were pooled and evaporated to 200 µl. Detection in all cases was by absorbance at 270 nm.

Quantitation of purified IQ and hydroxy-IQ by C_{18} HPLC (as described above) involved comparing the peak areas of the samples purified from faeces of each subject with those of synthetic standards. Peaks were integrated by an HP 3390A Recording Integrator. After quantitation, we pooled the respective IQ and hydroxy-IQ from each subject to have sufficient material for confirmation by TLC, UV spectroscopy and mutagenicity testing. The methods for obtaining TLC profiles on silica gel (chloroform/methanol/ammonia, 85:15:1) and UV spectra (260 nm to 340 nm) were as previously described.[7]

Salmonella/Microsomal Mutagenicity Assay

For mutagenicity testing we used a modification of the 'pre-incubation' method of the salmonella/microsomal mutagenicity test.[18] Tester strains of TA98 were grown from frozen stocks in 25 ml nutrient broth (Oxoid Ltd.) with vigorous shaking for 12 h at 37°C. S9 mix containing Arochlor 1254-induced rat liver S9 (Organon Teknika Corp.) was used for activation of compounds. Pre-incubation mixtures consisted of 0·3 ml S9 mix, 0·1 ml culture of tester strain and 0·1 ml of the test sample dissolved in DMSO; final concentration of S9 was 2 mg/ml. For complete details see Carman *et al.*[7] After 90 min at 37°C, we added each pre-incubation mixture to 3·5 ml soft-agar (55°C) and poured the mixture onto the minimal agar plates. All plates were incubated at 37°C for 48 h. All values are averages of duplicate plates.

Isolation and Characterisation of Hydroxy-IQ Producing Bacteria

Freshly passed faeces (1 g) from subject B were anaerobically inoculated into 10 ml of BHI broth supplemented with Tween-80, kanamycin and neomycin selective agents chosen on the basis of preliminary studies.[6,8] We incubated the broth at 37°C for 48 h, after which time 1 ml was transferred aseptically and anaerobically to a fresh Tween-80/kanamycin/neomycin broth and incubated as above. Following three more such serial transfers, the final culture was still able to convert IQ to hydroxy-IQ.[7]

The final culture was serially diluted (10-fold) and 0·1 ml from each dilution plated onto pre-reduced BHI agar which were incubated anaerobically at 37°C for 48 h. The plates from the highest dilution yielding discrete colonies on agar were replica plated; the replica plates were also incubated anaerobically at 37°C for 48 h. Following harvesting of all the colonies from the plate, the bacterial suspension was shown to convert IQ to hydroxy-IQ upon incubation in peptone yeast broth.[7] Smaller and smaller

Table 2. Comparison of IQ and hydroxy-IQ 'isolated' from faeces with 'synthetic' standards*

Assay	IQ		Hydroxy-IQ	
	Isolated	Synthetic	Isolated	Synthetic
TLC† (Rf)	0·54	0·54	0·28	0·28
HPLC‡ (k')				
Silica	4·5	4·4	8·5	8·6
C_{18}	7·8	7·7	3·2	3·2
TA98§ (−S9)	5	8	155	182
(+S9)	480	513	96	115

*Data reflects the values obtained with IQ and hydroxy-IQ preparations in which the respective compounds from each subject had been pooled.
†Rf = sample migration/solvent front migration. Both the IQ spots fluoresced yellow-brown, the hydroxy-IQ fluoresced blue-green.
‡k' = elution time − void time/void time.
§Values reflect [total revertants less spontaneous revertants] for 10 ng/plate of each compound. Spontaneous revertants; TA98 = 28 (−S9)/33 (+S9).

arcs of the plate were harvested until an area with a limited number of colonies was located. All the colonies in that area were isolated and tested for IQ conversion. Those producing hydroxy-IQ were identified using the methods for biochemical characterisation of Holdeman *et al.*[16] The ability of mixed and pure cultures of isolates to produce hydroxy-IQ from IQ *in vitro* was determined as previously described.[7]

RESULTS

Both IQ and hydroxy-IQ were present in blue cotton extracts of faeces of each of the subjects from the 30 h period immediately following ingestion of the fried meat meal. Sufficient amounts of each compound for characterisation by UV spectroscopy, TLC and HPLC and mutagenicity (Table 2) were isolated from the faeces. Neither IQ nor hydroxy-IQ in the normal 'pre-meat' and 'post-meat' faecal pools were detected.

Using HPLC, approximately 36 ng IQ (14 ng from subject A and 22 ng from subject B) and 29 ng hydroxy-IQ (12 from subject A and 17 from subject B) were purified from the faeces following the fried meat meal. This recovery correlates to a minimal level of IQ and hydroxy-IQ in the faeces of 0·05–0·08 ng/g and 0·03–0·07 ng/g faeces, respectively. We recovered 225 ng IQ from the fried meat control, or about 0·17 ng/g meat (pre-cooked weight); no hydroxy-IQ was detected. The detection limits both at 270 nm and 313 nm for IQ and hydroxy-IQ were 0·5 to 1·0 ng and 1 to 2 ng respectively.[7] The amount of each compound purified from faeces was sufficient to obtain useful UV spectra at the respective maxima; IQ, 260 nm and hydroxy-IQ, 270 nm and 313 nm. The spectrum for each compound purified from faeces was identical to that of its synthetic counterpart.

On TLC plates, the IQ and hydroxy-IQ purified from faeces had the same mobilities and distinctive fluorescence characteristics as the synthetic compounds (Table 2). Approximately 10 ng of each compound per spot were required for visualisation under long-wave UV light (360 nm). The IQ spots appeared yellow-brown and the hydroxy-IQ spots appeared blue-green. In order to obtain enough hydroxy-IQ for unequivocal mutagenicity results, we had to recover it after TLC analysis by extracting it from scraped spots with methanol/ammonia and re-isolating it by HPLC.[5,6]

On both normal and reverse phase analytical HPLC, the purified compounds had identical retentions as the synthetic compounds (Table 2). When we co-injected approximately 2 ng of each purified compound with 2 ng of its synthetic counterpart, they co-eluted as a single peak with twice the area of either compound alone.

The compounds isolated from faeces had the same mutagenic activities (Table 2) as their respective synthetic counterparts on which we previously reported.[7] The isolated IQ required microsomal activation for activity on TA98. Conversely, the isolated hydroxy-IQ was direct-acting on TA98 and inactivated by S9 microsomes. The mutagenic activities observed were consistent with the activities

one would expect with the amounts purified and assayed.

When faeces of each donor were diluted with anaerobic fluids and supplemented with IQ, both were capable of metabolising IQ to hydroxy-IQ within 3 d. From faeces of one donor, we isolated an organism (Gram-positive rod) which was similar to the 'lab' strain' *Eubacterium moniliforme* VPI 13480 which we have previously characterised.[7,8] The faecal isolate produced hydroxy-IQ from IQ in the characteristically reversible manner as *E. moniliforme* VPI 13480 when grown as pure cultures in broth media supplemented with IQ.

DISCUSSION

We were able to purify and quantitate IQ and hydroxy-IQ by HPLC using UV detection only because of the high extinction coefficients of these compounds, and the artificially elevated amounts present in the subjects' faeces. However, since extraction from faeces with blue cotton is at best 80–90 per cent,[15] the amounts recovered from faeces do not accurately reflect the total amounts present following the meal of fried meats. Accurate quantitation is further confounded by the fact that IQ, and presumably hydroxy-IQ, bind to colonic contents such as dietary fibre as well as to bacterial cells.[1,3,28] By having the subjects fast prior to and after eating the fried meats we hoped to minimise the potential for binding of the compounds to other dietary components.

Blue cotton extracted a number of other compounds from faeces, as indicated by multiple spots and peaks upon TLC and HPLC, respectively. Some of these were likely other major pyrolysis compounds such as MeIQ and MeIQx and their bacterial products, hydroxy-MeIQ and hydroxy-MeIQx. However, MeIQ, MeIQx, hydroxy-MeIQ and hydroxy-MeIQx elute from HPLC with considerably different retentions than IQ or hydroxy-IQ (Van Tassell, unpublished data).[11] We could detect none of these other compounds in our IQ or hydroxy-IQ preparations purified from faeces. We are currently studying the conversion of MeIQ and MeIQx to their hydroxy forms in faeces and by pure cultures of bacteria. We are also synthesising and characterising them with respect to their genotoxicity.

There is considerable variability in the amounts of IQ which are reported to be present in fried meats and faeces.[12,15,26] The levels of IQ we isolated from the fried meats and faeces are higher than those reported elsewhere, presumably because of the way in which the meats were fried and the high amounts consumed. Nevertheless, even at these artificially elevated levels, their concentrations are only of the order of parts per billion. Subsequently, structural analysis by NMR or mass spectrometry was not possible—there was not enough material. However, this is not to say that there is not enough of these compounds present in faeces to be of concern, as these are some of the most potent genotoxins which may be found in human intestines.

From the faeces of one subject a strain of bacteria which converted IQ to hydroxy-IQ, was isolated which conformed to the description of *Eubacterium moniliforme*. As we reported previously,[8] the organism was in relatively low numbers and required exhaustive techniques to isolate. Subsequently we concentrated on developing our techniques on faeces from the subject from which the most hydroxy-IQ was isolated. We are currently trying to isolate more strains and characterise them by immunological techniques using anti-eubacterial and anti-clostridial antisera.

Until the carcinogenic potential of hydroxy-IQ in experimental animals is determined, the importance of our current findings remains speculative. However there are possible implications to the *in vivo* activation of a dietary carcinogen to a direct-acting genotoxin by the colonic flora. Although hydroxylation of IQ occurring in diluted faeces *in vitro* can require 3–5 d, this is not necessarily the case *in vivo*. We have shown that, despite the relatively short transit times within the bowel, when IQ reaches the colon, the microflora will convert much of it to the direct-acting hydroxy-IQ. Even at the low concentrations observed, the hydroxy-IQ formed by the faecal flora *in vivo* might act directly on the mucosal cells of the colon or other organ sites, to initiate tumor formation. Activation by the liver through enterohepatic circulation would not be required. We are currently trying to determine if hydroxy-IQ causes organ site tumours in experimental animals.

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