

METABOLISM OF A HETEROCYCLIC AMINE COLON
CARCINOGEN IN YOUNG AND OLD RATS

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Non-Standard Abbreviations:

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

BNF: beta-naphthoflavone

HCA: heterocyclic amine

ESI: electrospray ionization

MS: mass spectrometry

CAD: collisionally activated dissociation

Abstract

Colon cancer increases with age, and this may be related to altered metabolism and deposition of carcinogens. One such carcinogen implicated in colon cancer is the heterocyclic amine found in well-done meat, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). The purpose of these studies was to determine whether the deposition and metabolism of IQ changes with age, comparing young (3 month) and old (22-24 month) male F344 rats. Animals were treated with vehicle or beta-naphthoflavone (BNF), an inducer of drug-metabolizing cytochrome P450s. Deposition and metabolism of IQ were determined after ip injection of radiolabeled IQ. Urinary IQ metabolites were identified and quantitated by HPLC and mass spectroscopy. In BNF-treated animals, total radiolabeled IQ excretion by old rats was less than half that of young. Binding of radiolabeled IQ metabolites by the old kidney was 10 times higher than the young. There were no age differences in intestinal and hepatic binding. There was a significant age-related increase in IQ conjugation to glucuronic acid and a decrease in conjugation to sulfate regardless of treatment. The induction of renal CYP1A1, a major P450 involved in IQ metabolism, by BNF did not change with age. Changes in IQ metabolism with age along with altered renal function may contribute to the decreased urinary excretion and increased renal binding of IQ and/or its metabolites seen in the old animals.

The incidence of most cancers, such as colon cancer, increase exponentially with age (Jemel, 2005). The reasons for this age-dependence are complex, but altered metabolism of carcinogens with age can play a role. One such carcinogen is 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (see Figure 1), a heterocyclic amine (HCA) found in well-done red meat. IQ is one of seventeen new entries in the 2005 Eleventh Report on Carcinogens (NTP, 2005). IQ is a potent carcinogen in rodents (Sugimura,2000;Tanaka,1985;Takayama,1984) and non-human primates (Adamson,1990). In rodents, IQ induces tumors in liver, small and large intestine, pancreas, lung, and mammary gland. In humans, metabolites of IQ have been found in urine (Lynch,1992), and HCAs have been implicated in the initiation of colon cancer (Kristiansen,1997). This suggests that the strong association between well-done red meat and colorectal cancer risk (Giovannani,1994) may be mediated by HCAs such as IQ.

The biotransformation of IQ in young rats has been well studied. Important metabolites of IQ which have been identified are shown in Figure 1. An important pathway is ring oxidation at the 5 position of IQ mediated by cytochrome P450 and followed by conjugation with sulfate or glucuronic acid (Luks,1989;Inamasu,1989). The other major route of metabolism is direct conjugation of the exocyclic amine by N²-glucuronidation or sulfamate formation (Luks,1989;Inamasu,1989;Turesky,1986). Sulfamate excretion was observed in urine, bile, and feces (Luks,1989;Inamasu,1989;Turesky,1986). In contrast, the 5-O-glucuronide and 5-sulfate were not detected in feces (Luks,1989;Inamasu,1989). This is consistent with the conjugates being hydrolyzed by bacterial enzymes in the intestine and re-absorbed from the intestinal tract. Despite nearly complete detoxification of IQ by the rat, a significant amount of oxidation of IQ appears to occur, forming DNA adducts and contributing to the presence of tumors (Sugimura,2000;Tanaka,1985;Takayama,1984;Turesky,1995).

Although the metabolism of IQ has been well-studied in young animals, there have been no studies of the metabolism IQ in old animals. In general, the effect of age on metabolism depends on the compound being studied. For example, one dioxin compound (TCDD) shows greater concentrations in the tissues of old mice with less than 1% excreted (Pegram, 1995), while benzopyrene forms fewer adducts in old mice (Boerrigter, 1995).

The purpose of these studies was to determine whether the metabolism of IQ changed with age and with cytochrome P450 induction. Radiolabeled IQ was administered to young and old rats. The amount of radioactivity excreted in the urine and the urinary metabolite profile was determined. Since the cytochrome P450s play a major role in IQ metabolism, the effect of beta-naphthoflavone (BNF), an inducer of P450 metabolism, on IQ metabolism was also determined. Finally, as a possible indication of IQ adduct formation, the amount of IQ retained in specific tissues was determined as a function of age.

Methods

Materials. [2-¹⁴C]-IQ (50 mCi/mmol, >98% radiochemical purity) and IQ were purchased from Toronto Research Chemicals (Toronto, ON). Diethylenetriaminepentaacetic acid (DETAPAC), BNF, *E. coli* beta-glucuronidase (Type VII-A), and Abalone sulfatase (Type VIII) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Ultima-Flo AP was purchased from PerkinElmer LAS, Inc., Shelton, CT.

Animals and dosing. Young and old male F344 rats were purchased from Harlan, Indianapolis, IN. Young rats were 3 months old and old rats were retired breeders 22-24 months old. Food and deionized water were provided *ad libitum*. Animals were administered 20 mg/kg ¹⁴C-labeled IQ (20 uCi; 1.25 ml per 250 gm injected ip; 20% DMSO : 80% 0.1 N HCl), placed in metabolic cages, and a 24-hour urine collected. To induce hepatic phase I and II enzymes, these same animals were treated with 40 mg/kg BNF (2.5 ml per 250 gm injected ip; 13% DMSO : 87% corn oil) for 3 consecutive days. Rats were then administered 20 mg/kg ¹⁴C-labeled IQ ip, a 24-hour urine collected, and rats euthanized by cervical dislocation.

Processing urine and purification of metabolites. Urine was analyzed for IQ metabolites. The samples were treated with an equal volume of methanol and spun at 1,500 xg for 20 min to precipitate protein and debris. After evaporation of the methanol, the urine was applied to a Sep-Pak column, previously prepared with methanol/water, washed with 5 ml water, and eluted with 10 ml 100% methanol. The latter was concentrated by evaporating the methanol and reconstituted in 100 mM ammonium acetate buffer (pH 7.4). The resuspended material was extracted twice with 2 volumes of ethyl acetate and then with equal volumes of n-butanol three times. There was no significant radioactivity in the ethyl acetate extract. The combined n-butanol extracts were back extracted with an equal volume of pH 7.4 buffer, and the organic layer was evaporated under N₂ gas. The total recovery of radioactivity in the aqueous (37%) and n-butanol (48%) fractions was

85%. Urine from each rat was analyzed using solvent system 1 (see below). For identification of metabolites, urine from a young rat was purified using solvent systems 1, 2, and 3, respectively. After each purification step, metabolite peaks were concentrated and applied to the next solvent system for further purification.

HPLC analysis of products. Products were assessed using a Beckman HPLC with System Gold software and a 5 μ m, 4.6 x 150 mm C-18 ultrasphere column attached to a guard column. For solvent system 1, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in 2% acetonitrile, 0-5 min; 2-5% acetonitrile, 5-23 min; 5-40% acetonitrile, 24-29 min; 40-2% acetonitrile, 35-40 min; flow rate 1 ml/min. For solvent system 2, the mobile phase contained 20 mM ammonium acetate (pH 6.8) in 4% acetonitrile, 0-5 min; 4-5% acetonitrile, 5-23 min; 5-40% acetonitrile, 24-29 min; 40-4% acetonitrile, 35-40 min; flow rate 1 ml/min. For solvent system 3, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in 10% methanol, 0-15 min; 10-90% methanol, 15-20 min; 90-10% methanol, 25-30 min; flow rate 1 ml/min. Radioactivity in HPLC eluents was measured using a FLO-ONE (PerkinElmer LAS, Inc., Shelton, CT) radioactive flow detector. Data are expressed as a percent of total radioactivity or pmol recovered by HPLC.

Mass spectral analysis. Electrospray ionization (ESI) mass spectrometry (MS) analyses were performed on a Finnigan TSQ-7000 triple stage quadrupole mass spectrometer (San Jose, CA) equipped with a Finnigan ESI source and controlled by Finnigan ICIS software operated on a DEC alpha workstation. Samples were loop injected onto the ESI source with a Harvard syringe pump, which is continuously infused with methanol at a flow rate of 5 μ L/min. The skimmer was at ground potential, and the electrospray needle was at 4.5 kV. The heated capillary temperature was 250⁰C. To obtain collisionally activated dissociation (CAD) tandem mass spectra, the collision energy was set at 22 eV, and argon (2.3 mTorr) was used as target gas. The product ion spectra were acquired in the profile mode at the scan rate of one scan per 3 sec.

Qualitative identification of urinary metabolites. To assist in identifying IQ metabolites in individual urine samples, their elution time and susceptibility to specific treatments were determined (Luks,1989;Inamasu,1989;Turesky,1986). The 5-O-glucuronide is susceptible to *E. coli* beta-glucuronidase (Type VII-A, Sigma, St. Louis, MO) with 100 units enzyme at pH 6.8 and 0.3 ug substrate per 0.1 ml at 37 °C for 24 hours. 5-Sulfate is susceptible to Abalone sulfatase (Type VIII, Sigma) with 10 units at pH 5.0 and 0.3 ug substrate per 0.1 ml at 37 °C for 24 hours. N²-Glucuronide and sulfamate are hydrolyzed with 1 N HCl at 60 °C for 4 hr.

Measurement of CYP1A1 protein levels. Rat liver, duodenum and renal cortex were homogenized (150 mg tissue/ ml) in ice cold homogenizing buffer (25 mM NaCL, 1mM Tris, pH 8.0). Protein levels of CYP1A were measured by Western blotting with chemiluminescence detection as previously described (Armbrecht, 1999). The polyclonal antisera used was CYP1A1 (G-18): sc-9828 from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). This antisera recognizes CYP1A1 from mouse, rat, and human. Homogenate proteins were separated out by SDS-PAGE using 10% gels. Separated proteins were transferred to Hybond-P membranes (Amersham-GE Healthcare, Piscataway, NJ), and membranes were incubated for 1 hours with 1:1000 of the primary antisera followed by 30 minutes with 1:20,000 of the secondary antisera – donkey anti-goat IgG-HRP (Santa Cruz). Antigen-antibody complexes were visualized using an ECL Western blotting kit and ECL Plus Hyperfilm (Amersham-GE Healthcare). Bands were quantitated by optical densitometry using an Amersham Biosystems ImageScanner. Based on actin rehybridization, sample loading was quite uniform. Therefore, absorbance was routinely normalized to total protein.

Statistical Analysis. The data from these experiments are reported as means \pm SE of the number of animals indicated. Experiments with a 2x2 design (age vs BNF treatment) were first analyzed by two-way ANOVA. If needed, individual comparisons were performed using Student's two tailed t-test. A confidence level of 95% or greater was considered significant.

Results

Effect of age and BNF on excretion of radiolabeled IQ metabolites. Control and BNF-treated rats of each age group were injected with radiolabeled IQ based on body weight. A 24-hour urine collection was performed, and the total amount of radioactivity excreted, expressed as a per cent of the injected dose, was calculated (Figure 2). In control rats, there was no change in excretion with age with 54-55% of the dose being excreted. In BNF-treated rats, the amount excreted dropped to 31% in young rats and to 15% in old rats. The amount excreted in the old rats was significantly less than that in the young rats. To determine the mechanisms responsible for age difference, the IQ metabolites in the urine were identified and quantitated.

Analysis of IQ metabolites in rat urine by HPLC. The HPLC profile of IQ urinary metabolites from untreated rats is illustrated in Figures 3 and 4 (upper panels). Peaks eluting at 15.0, 20.1, 22.8, and 28.5 min were tentatively identified as N-glucuronide, 5-O-glucuronide, sulfamate, and 5-sulfate, respectively in both age groups (Figures 3 and 4, upper panels). The metabolite profile was changed after administration of BNF. Major peaks were only observed for 5-O-glucuronide and 5-sulfate (Figures 3 and 4, lower panels). Positive peak identity was determined by either ESI CAD tandem mass spectrometry or by their elution time and susceptibility to specific treatments.

Identification of IQ metabolites by tandem mass spectrometry. Urinary IQ metabolites from a young rat were purified by HPLC, utilizing 3 different pH and solvent conditions. Metabolites were identified by ESI/MS in both the positive- and negative-ion modes. In the positive-ion mode, the product at 20.1 min exhibited $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ ions at m/z 391, 413, and 429, respectively. In negative-ion mode, the product yielded deprotonated $[M - H]^-$ molecular ions at m/z 389 and gave rise to prominent ions at m/z 213 and 198, representing 5-OH-IQ and loss of CH_3 . A neutral loss of 176 is the characteristic product ion of glucuronides. The 20.1 min product is IQ-5-O-glucuronide. The 22.8 min peak, in the negative mode, exhibited an $[M - H]^-$ ions at m/z 277

and gave rise to prominent ions at m/z 197 and 182, representing IQ and loss of CH_3 . The ion at m/z 80 corresponded to SO_3 . The 22.8 min product is the IQ-sulfamate. In the negative mode, the 15.0 min peak exhibited an $[\text{M} - \text{H}]^-$ ions at m/z 373 and gave rise to prominent ions at m/z 197 and 182, representing IQ and loss of CH_3 . The 15.0 min product is the IQ- N^2 -glucuronide. For the 28.5 min peak, the negative mode exhibited an $[\text{M} - \text{H}]^-$ ions at m/z 293 and gave rise to prominent ions at m/z 213 and 198, representing 5-OH-IQ and loss of CH_3 . The ion at m/z 80 corresponded to SO_3 . In the positive-ion mode, the product yielded $[\text{M} + \text{Na}]^+$ at m/z 317. This fragmentation pattern is consistent with the 28.5 min peak being IQ-5-sulfate. These metabolites were present in both the aqueous and n-butanol fractions. Results are consistent with previous studies of IQ metabolites in rat (Luks,1989;Inamasu,1989;Turesky,1986).

Identification of IQ metabolites by susceptibility to specific treatments. To assist in identifying IQ metabolites in individual urine samples, the Sep Pak purified fraction was subjected to specific enzyme or acid treatment and then analyzed by HPLC. The N-glucuronide was susceptible to treatment with 1 N HCl at 60 °C, 5-O-glucuronide to beta-glucuronidase, sulfamate to 1 N HCl at 60 °C, and 5-sulfate to sulfatase. Metabolites were only susceptible to the treatments indicated. Because of the small amounts of N-glucuronide and sulfamate present in urine from BNF-treated rats, these apparent metabolites were not further identified. These results are consistent with their ESI/MS assigned structures by above.

Effect of age and BNF on urinary IQ metabolites. The metabolites identified in Figures 3 and 4 were quantitated by HPLC (Table 1) and analyzed statistically by two-way ANOVA (Table 2). BNF treatment had a highly significant effect in both age groups. It increased 5-O-glucuronide 200-286% and increased 5-sulfate by 48-56% in both age groups. This is consistent with the fact that BNF increases both phase 1 (P450s) and phase 2 (UDP-glucuronosyltransferases) enzymes. Likewise, it decreased N-glucuronide to 18-22% of control and sulfamate to 2-3% of control in both

age groups. With age there was a significant increase in 5-O-glucuronide and significant decrease in 5-sulfate (Table 2). This indicated a significant metabolic shift in older animals from 5-sulfate to 5-O-glucuronide metabolism. There was no difference in the effect of BNF with age.

Effect of age on IQ metabolites in tissue. After urine collection, the BNF-treated rats were euthanized and the tissues were assessed for the distribution of radioactivity (Figure 5). Levels of radioactivity were similar in the intestine and liver of the two age groups, but the amount of radioactivity in the kidneys of the old rats was dramatically higher than that of the young. Because of the large amount of radioactivity in the old kidneys, the radioactivity present was further characterized (Figure 6). The renal cortical homogenates from 3 old rats were mixed with an equal volume of methanol, forming a precipitate. The supernatant of this was precipitated with 5% TCA. The total precipitates (methanol and TCA) contained $31 \pm 0.7\%$ of the radioactivity and the soluble fractions contained $66 \pm 7\%$. The precipitates were pooled and incubated with proteinase K for 3 hr at 60°C . Following this treatment, 33% of this material was now soluble. The soluble fractions remaining after the TCA precipitation ($66 \pm 7\%$ of the total) were pooled and applied to a C18 solid-phase extraction column (1 gm, PrepSep) for further purification (Figure 6). Of this material, 61% was eluted from the column while 39% was retained. HPLC analysis of the elutant indicated that it contained 60% 5-O-glucuronide and 24% 5-sulfate. Treatment with beta-glucuronidase and sulfatase confirmed the identification of these metabolites. This distribution of metabolites in renal tissue is quite similar to the percentages seen in urine from old, BNF-treated rats (Table 1).

Effect of BNF and age on expression of CYP1A1. The effect of BNF on CYP1A1 expression was determined in the liver and kidney (Figure 7). CYP1A1 protein levels were undetectable in the absence of BNF in both tissues and age groups. BNF markedly increased CYP1A1 protein levels in the liver and the kidney. There was no significant difference with age in either tissue.

Discussion

These studies demonstrate changes with age in both the metabolism and deposition of IQ. In the young rat, the primary metabolism of IQ is via direct conjugation to sulfate (sulfamate) (49%) (Table 1). BNF markedly induces CYP1A1 expression (Figure 7) and shifts metabolism of IQ to the P450-mediated pathway (5-O-glucuronide and 5-sulfate) and away from sulfamate (1.3%). IQ has been shown to be metabolized by human CYP1A1 and 1A2, although to a much greater extent by CYP1A2 (McManus,1990). However, of relevance to these studies, BNF increases CYP1A1 activity to a greater degree than 1A2 activity in rodents (Turesky, 1998). Preliminary studies with liver slices from young rats have demonstrated almost complete blockage of 5-O-glucuronide and 5-sulfate formation by ellipticine, a CYP1A1 inhibitor (data not shown). This supports the metabolism of IQ by CYP1A1 in the rat.

In the old rat there is a significant increase in 5-O-glucuronide and decrease in 5-sulfate compared to young rats (Table 1). BNF markedly increases 5-O-glucuronide and decreases 5-sulfate in both age groups, but there is no significant change in the effect of BNF with age (Table 2). This suggests that there is no change in the P450 metabolic pathway with age either basally or after BNF stimulation. This is supported by the fact that the induction of CYP1A1 protein by BNF does not change with age in the liver or kidney (Figure 7). The major age-related change in BNF metabolism is an increase in glucuronic acid conjugation and a decrease in sulfate conjugation.

A major finding of this study is that there was decreased excretion and increased renal binding of IQ metabolites with age. The amount of radiolabeled IQ retained by the old kidney is much higher than in the young kidney or any other tissue regardless of age (Figure 5). When this radioactivity was further characterized (Figure 6), it was found that 31% was precipitable and 26% was retained by the C18 column. Thus 57% of the radioactivity probably represents metabolites bound to macromolecules in the kidney. Only 24% was recovered as soluble IQ-5-O-glucuronide and 10%

as IQ-5-sulfate. These findings suggest that much more of the IQ metabolites are complexed with proteins and nucleic acids in the old kidney compared to the young kidney. However, even in the old kidneys, the amount of radioactivity bound is a very small percentage of the total dose administered. Binding itself would not explain the decreased radiolabeled IQ excretion by the old animals. It is of interest that 3-methylcholanthrene also decreased urinary excretion of a heterocyclic amine carcinogen in young mice (Buonarati, 1992). In this study, 3-methylcholanthrene increased binding in the liver but decreased binding in the kidney.

One possible explanation for a sharp increase in IQ binding in the old rat kidney is an age-related decrease in antioxidant defenses. In the F344 rat kidney, there is an age-related decrease in glutathione and glutathione transferase activity (Liu,2003) and in superoxide dismutase and catalase activity (Tian,1998). This leads to an increase in reactive oxygen species in the old rat kidney that manifests itself in a number of ways. The formation of protein carbonyl groups (Greenberg,2000) and advanced glycation end (AGE) products (Teillet,2000) increases with age in the kidney. Renal telomere shortening, due in part to free radical damage, and an increase in cell senescence markers have been observed in the old kidney (Tarry-Adkins, 2006). Of direct relevance to the present study, there is an increase in DNA adducts (8-hydroxy-2'-deoxyguanosine) in the F344 rat kidney with age (Hamilton, 2001). Thus, the environment of the old kidney may favor the binding of IQ metabolites. There are also physiological changes in the old rat kidney, such as decreased glomerular filtration rate, which may contribute to decreased excretion, increased metabolite exposure, and increased binding to macromolecules (Baylis, 1998). Conversely, this increased binding to macromolecules may, in turn, contribute to physiological changes with age.

It is of interest that there is no change in the induction of CYP1A1 by BNF with age (Figure 7). In general, the effect of age on the induction of the hepatic P450 xenobiotic-metabolizing pathways is complex. In a series of experiments, Horbach et al. administered multiple doses of various

inducing agents i.p. to rats and then measured P450 induction at the level of mRNA and protein. At the mRNA level, a number of differences in induction with age were found. Induction of CYP1A2, CYP2B1 and CYP2B2 by isosafrole declined with age (Horbach,1990a; Horbach,1990b). Induction of CYP2B1 and CYP2B2 by pentobarbital also declined with age (Horbach,1990a). However, at the protein level there was no difference in the maximal levels of P450 protein induced as a function of age (Horbach,1992). In another study, low doses of pentobarbital showed no effect of age in terms of inducing a whole range of hepatic P450s (Agarawal, 2003). The capacity of TCDD to induce some forms of P4501A either remained unchanged or increased with age (Pegram, 1995). In the present studies, the fact that the BNF induction of CYP1A1 protein does not change with age would be consistent with previous aging studies.

These studies in the rat raise the possibility that the metabolic products of some carcinogens may accumulate preferentially in tissues of older animals. This may occur without major changes in the metabolism of those carcinogens with age. This is supported by a study of the effect of age on the distribution of a single oral dose of a dioxin compound (TCDD) (Pegram, 1995). In this study, the blood concentrations of TCDD did not change with age, but the TCDD concentrations in skin, kidney, and muscle in old mice were twice those of the young. On the other hand, after a single intraperitoneal dose of benzopyrene, there were significantly fewer adducts formed in organs of old mice compared to young mice (Boerrigter, 1995). Thus, the effect of age may be specific to a particular compound and tissue.

In the human diet, consumption of 400 gm of cooked lean meat could result in exposure to several micrograms of mutagenic heterocyclic amines (Pais,1999), which have been detected in urine (Lynch,1992), indicating their absorption from cooked foods. The strong association of high temperature cooked meat intake and colorectal cancer risk (Giovannucci,1994), the presence of heterocyclic amines in cooked meat (Pais,1999), and the initiation of colon cancer by heterocyclic

amines (Kristiansen,1997), suggest that heterocyclic amines present in high temperature cooked meat may be responsible for the increased risk of colon cancer associated with this dietary component. The present studies should further our understanding of IQ-induced colon cancer.

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Footnotes

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Figure Legends

Figure 1 – Structures of IQ and its Metabolites.

Figure 2 – Effect of Age and BNF on Urinary Excretion of ¹⁴C-IQ. Urine from young (3 month old) and old (22-24 month old) rats was collected for 24 hours after i.p. injection of radiolabeled IQ. Radioactivity in the urine was determined by scintillation counting and is expressed as a per cent of the total dose administered. Bars are the mean \pm SE of 4 rats.

^aSignificantly different than Control of same age group (P<0.05, t-test)

^bSignificantly different than Young of same treatment group (P<0.05, t-test)

Figure 3 – HPLC Profile of Urinary IQ Metabolites from Young Rats. Rat urine from control (top) and beta-naphthoflavone (β -NF) (bottom) rats was extracted and applied to a C-18 ultrasphere column and eluted with solvent system 1. The peaks were tentatively identified by retention times and confirmed by mass spectrometry and susceptibility to specific treatments. A representative profile from each treatment group is shown.

Figure 4 – HPLC Profile of Urinary IQ Metabolites from Old Rats. Experimental details are the same as Figure 3.

Figure 5 – Effect of Age on Tissue Binding of Radiolabeled IQ. The radioactivity in tissue homogenates from young and old rats treated with BNF was determined by scintillation counting. Radioactivity from duodenum (Int), kidney cortex (Kid) and liver (Liver) is expressed as cpm per mg of total protein. Bars are the mean \pm SE of 4 rats.

^aSignificantly different than young (P<0.05, t-test)

Figure 6 – Purification Scheme for Renal Cortex Homogenate. To further characterize the radioactivity found in old kidneys (see Figure 5), the kidney homogenates were further purified (see Methods for details). The numbers in parentheses are the mean \pm SE of the per cent radioactivity found in each fraction or sub-fraction.

Figure 7 – Effect of Age and BNF on CYP1A1 Protein Levels. CYP1A1 protein levels were determined in liver and renal homogenates in young and old rats with and without BNF. Protein levels were determined by Western blotting (see Methods for details) and are expressed as a percent of the young BNF-treated levels for each tissue. Bars are the mean \pm SE of 4 rats (top). BNF markedly increased CYP1A1 levels in both tissues but there was no effect of age. A Western blot of pooled samples from BNF-treated animals is shown at bottom (L = liver, K = kidney, O = old, Y = young). Numbers along side are molecular weight markers in kilodaltons.

Table 1 - Effect of BNF Treatment and Age on Urinary IQ Metabolites

Condition	N-Glucuronide	5-O-Glucuronide	Sulfamate	5-Sulfate
% of Total Urine Radioactivity				
<u>Young Rats</u>				
Control	6.0 ± 1.0	14 ± 2	49 ± 2	21 ± 1
BNF-Treated	1.1 ± 0.6	54 ± 4	1.3 ± 1.0	31 ± 2
<u>Old Rats</u>				
Control	6.5 ± 0.7	21 ± 4	44 ± 5	16 ± 2 ^a
BNF-Treated	1.4 ± 0.9	63 ± 1 ^a	0.9 ± 0.5	25 ± 1 ^a

Table entries are the mean ± SE of 4 rats. Rats were administered radiolabeled IQ and placed in metabolic cages for a 24-hr urine collection. These same rats were then administered BNF (40 mg/kg) for three consecutive days and again administered radiolabeled IQ with a second 24-hr urine collected. Young and old rats were 3 and 24-26 months old respectively.

^aSignificantly different than Young of same age and treatment group (P < 0.05, t-test).

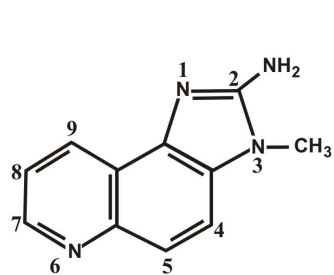
Table 2 - Two-Way ANOVA of BNF Treatment and Age

	<u>N-Glucuronide</u>	<u>5-O-Glucuronide</u>	<u>Sulfamate</u>	<u>5-Sulfate</u>
BNF	0.001*	0.001*	0.001*	0.001*
Age	0.576	0.016*	0.297	0.003*
Age x BNF	0.778	0.594	0.361	0.802

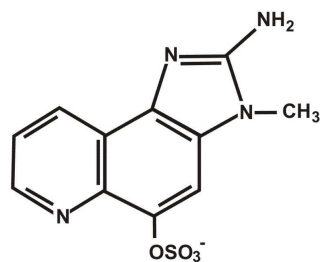
* Statistically significant effect of BNF and/or Age (P<0.05).

FIGURE 1

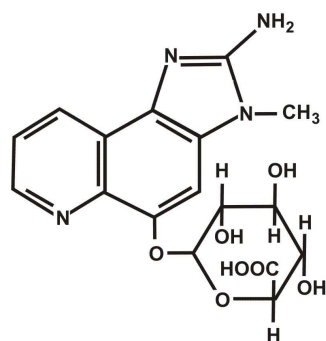
Structures of IQ and Its Metabolites



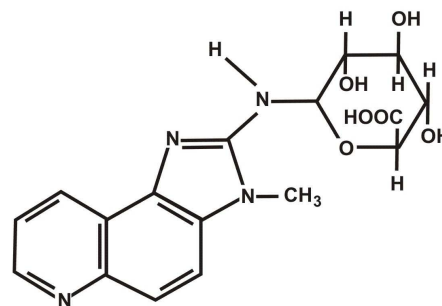
IQ



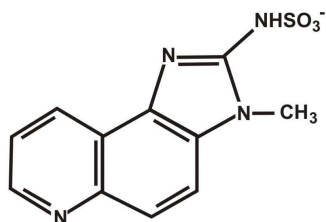
IQ-5-sulfate



IQ-5-O-glucuronide

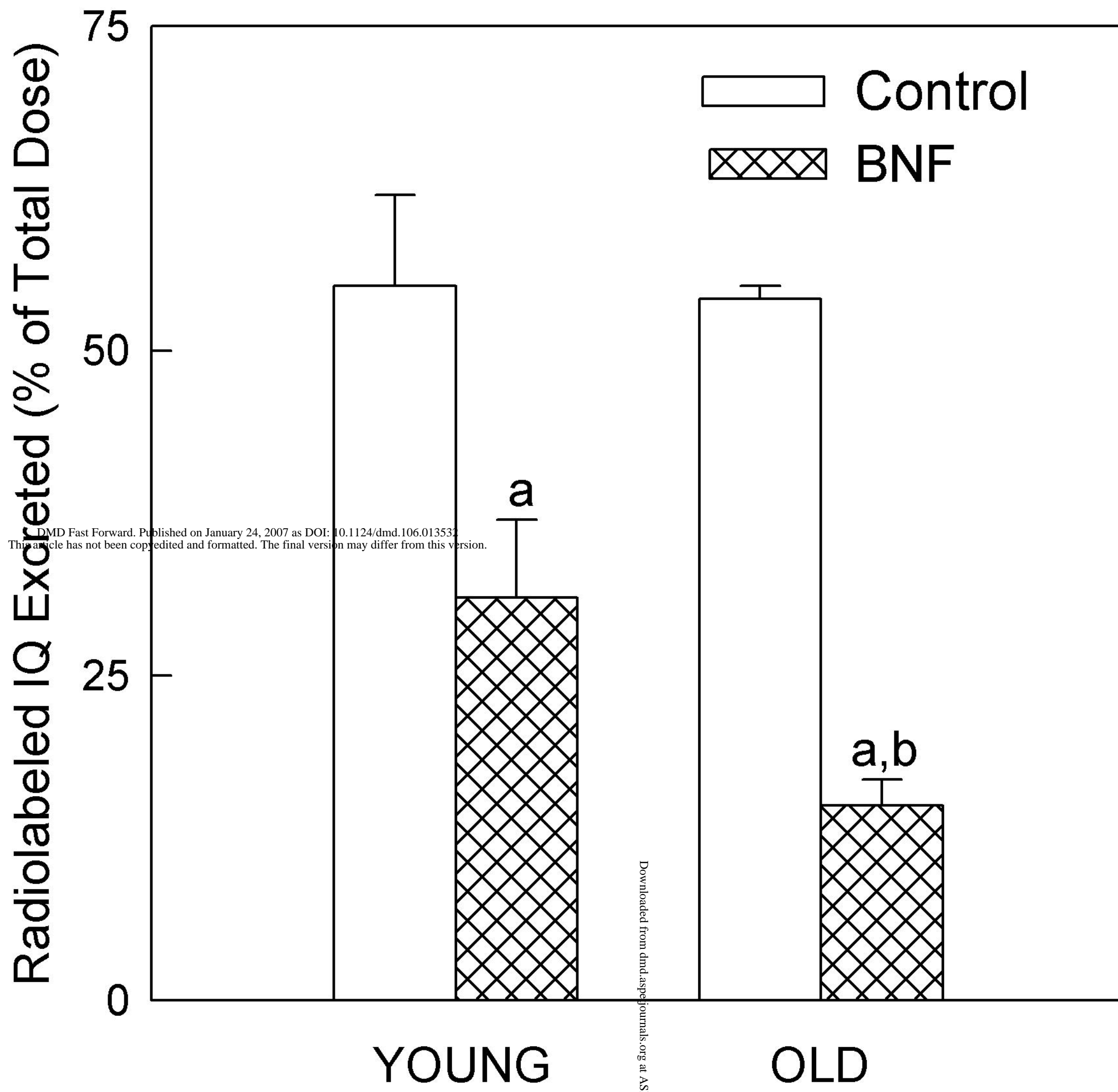


IQ-N²-glucuronide



IQ-sulfamate

FIGURE 2



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FIGURE 3

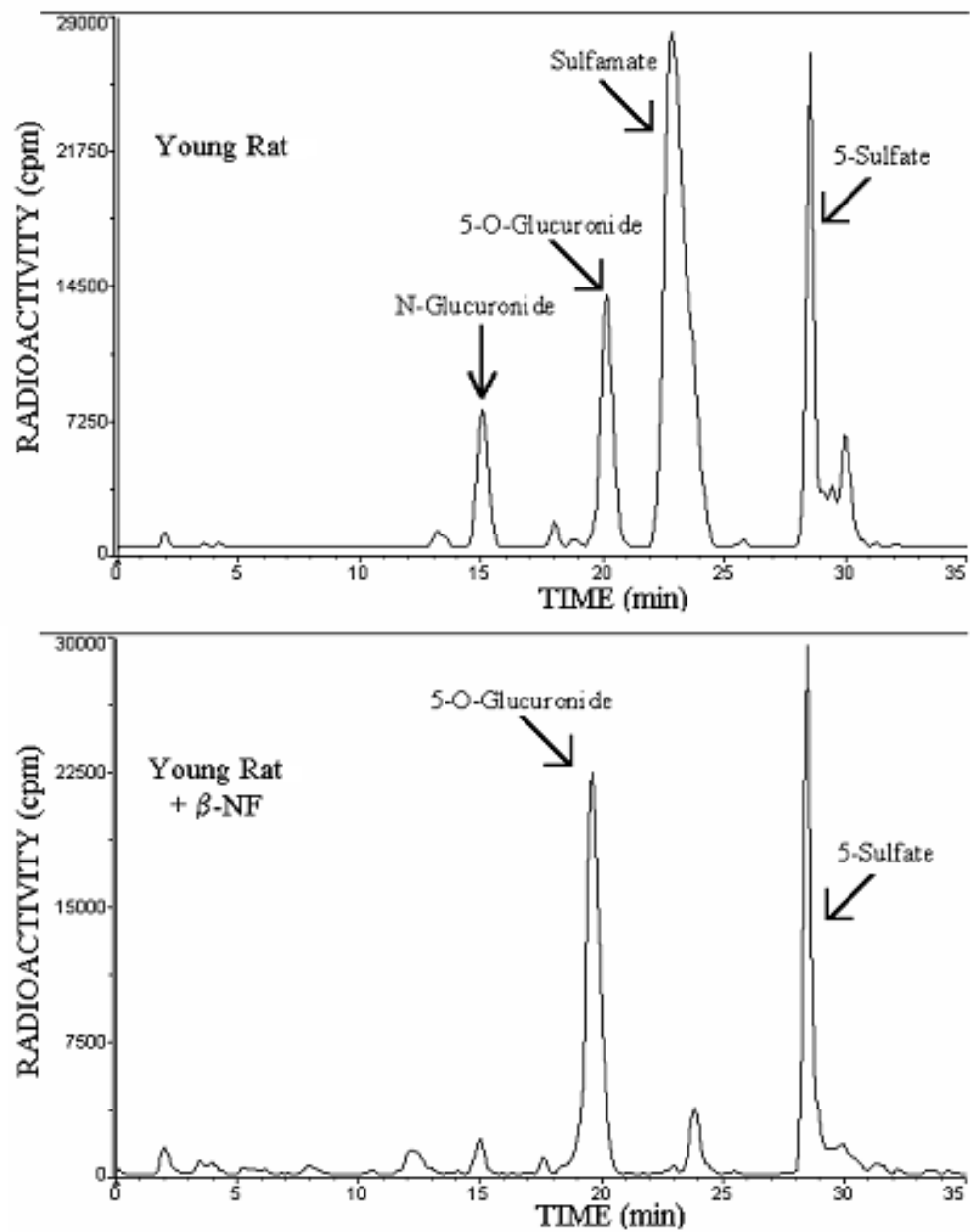


FIGURE 4

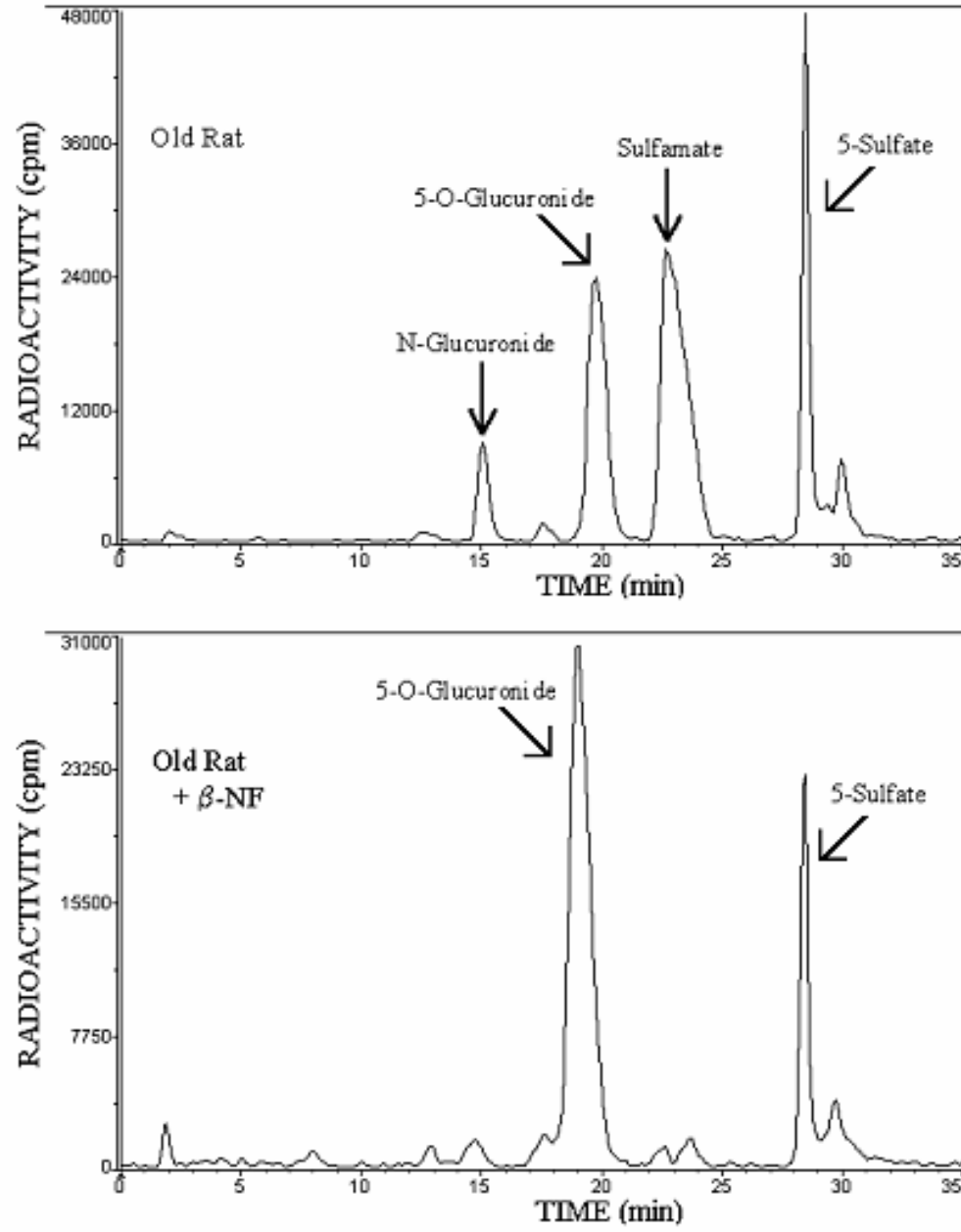
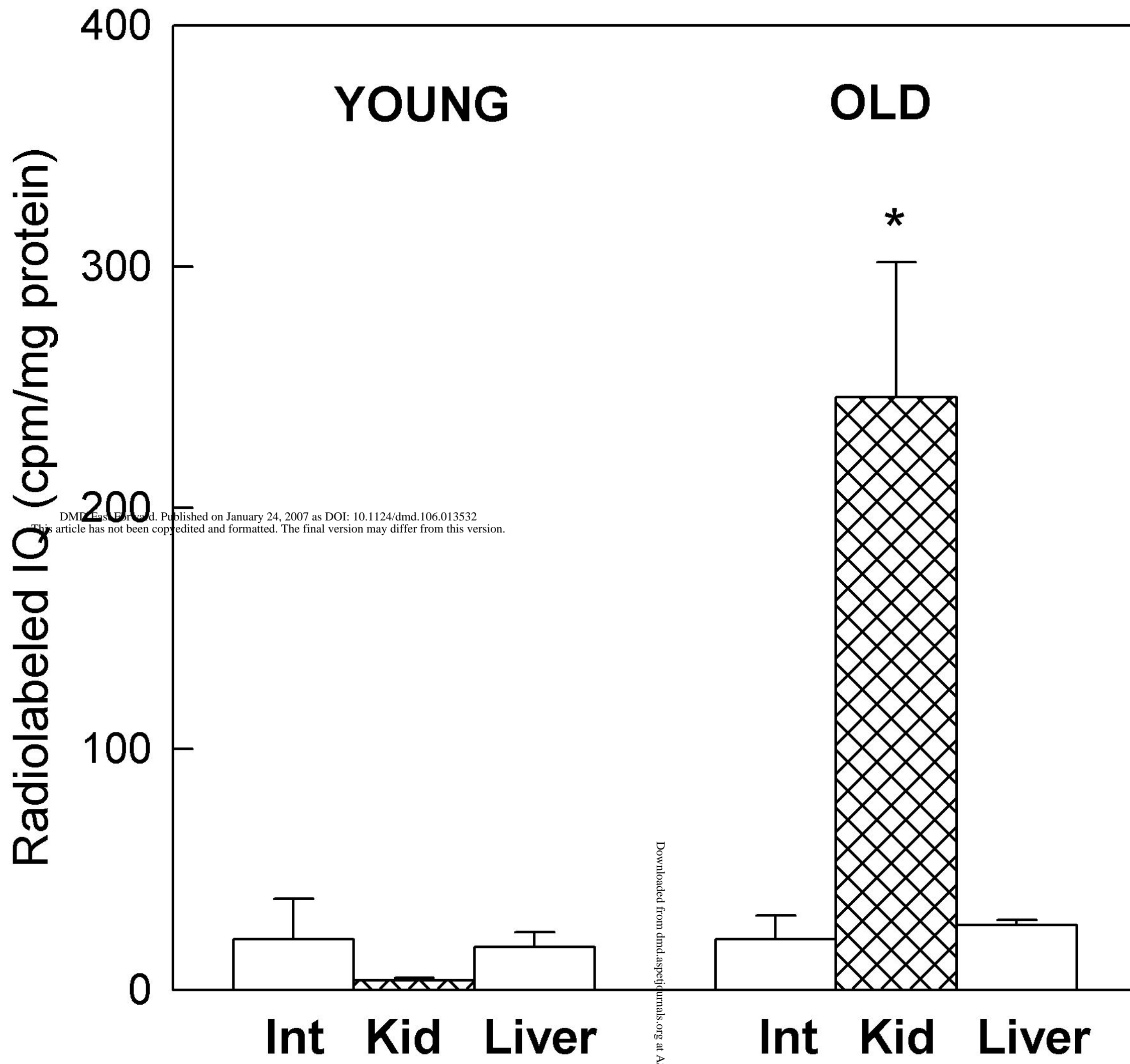


FIGURE 5



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FIGURE 6

Whole Renal Cortex Homogenate Purification Scheme

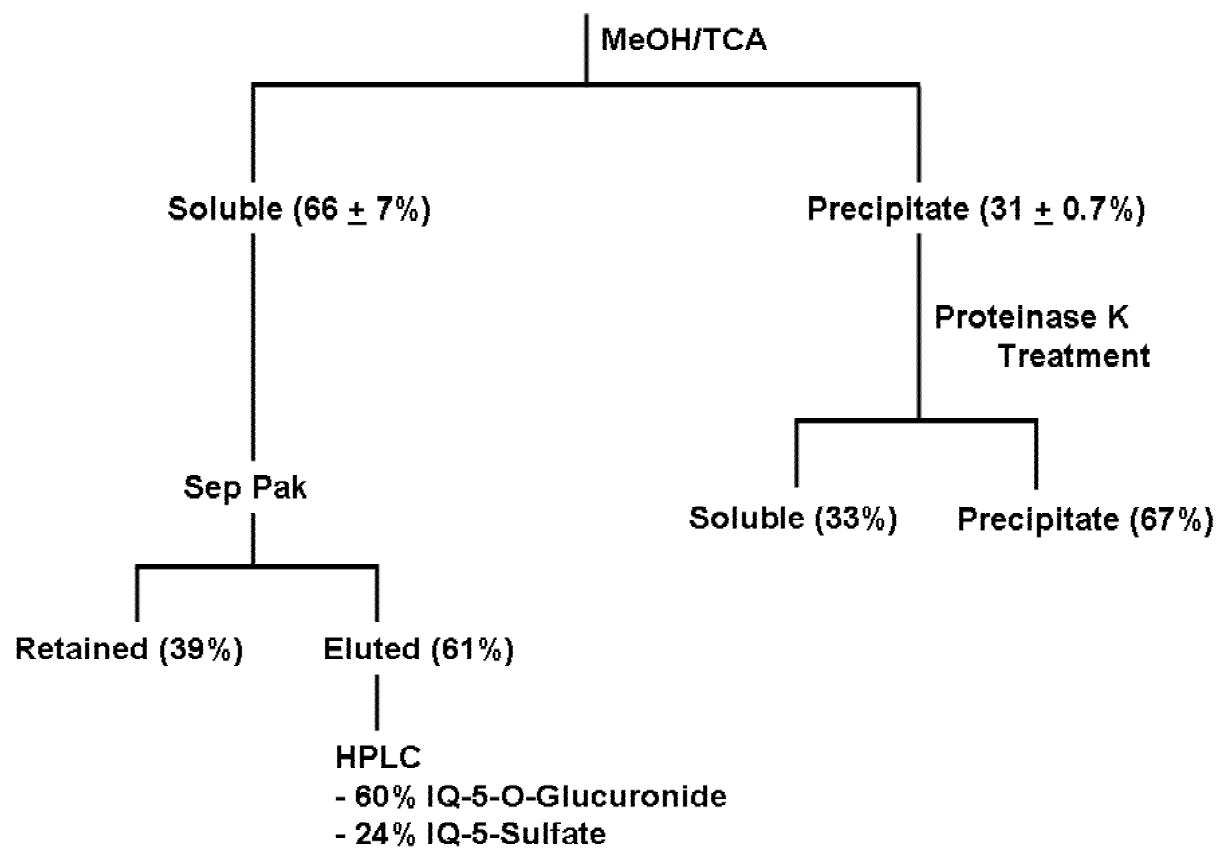


FIGURE 7

