starchy foods but probably not those which occur in meats.

Registry No. 1, 72-19-5; 2, 672-15-1; 3, 352-21-6; 4, 156-87-6; 5, 78-96-6; 6, 80-60-4; 7, 541-48-0; 8, 56-12-2; 9, 62-57-7; 10, 300-85-6; 11, 591-81-1; D-glucose, 50-99-7; starch, 9005-25-8.

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Microbial Metabolism of [^{14}C]Nitroanilines to [^{14}C]Carbon Dioxide

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A strain of *Pseudomonas* (P6), isolated from soil, grew slowly on *p*-nitroaniline (PNA) as a sole source of carbon. PNA degradation was considerably faster in the presence of yeast extract. A culture grown on 1.5 mM [^{14}C]PNA plus 200 ppm of yeast extract for 8 days released 73% of the initial radioactivity as $^{14}CO_2$ and 16% of the radioactivity was associated with the cells. *o*-Nitroaniline (ONA) and *m*-nitroaniline (MNA) were not degradable as sole sources of carbon. Strain P6, however, was able to degrade [^{14}C]MNA rapidly to $^{14}CO_2$ in the presence of PNA.

Nitroanilines and their derivatives occur in waste water from the dye and pharmaceutical industries and in soils as metabolites from microbial degradation of certain herbicides (Golab et al., 1979; Laanio et al., 1973; Leipzig and Hockenbury, 1979; Stephenson et al., 1979). Literature on the microbial degradation of nitroanilines is very limited. Alexander and Lustigman (1966) incubated 5–10 ppm of *o*-nitroaniline (ONA), *m*-nitroaniline (MNA), and *p*-nitroaniline (PNA), respectively, with soil suspensions and found no significant degradation within 64 days. Young and Affleck (1974) incubated 50 ppm of ONA, MNA, and PNA, respectively, with suspensions of sewage sludge and monitored the biochemical oxygen demand (BOD). The BOD indicated a slow degradation of PNA within 60 days, but no degradation of ONA and MNA was found. Partial degradation of MNA took place within 60 days in the presence of PNA. No direct chemical analysis was performed to verify the degradation of PNA and MNA.

Total degradation of nitroanilines by pure cultures of microorganisms has not been previously reported. Only reduction of the nitro substituent leading to the accumulation of the corresponding diamino derivative or coupling reactions involving the amino substituent were found to occur in pure cultures (Lusby et al., 1980; McCormick et al., 1976).

In contrast to the nitroanilines, microbial degradation of aniline and nitrobenzene derivatives has been well documented. Aniline and aniline derivatives such as *m*-

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chloroaniline and *p*-chloroaniline are degraded rapidly through catechol intermediates and the amino substituent released as ammonia (Bachofer et al., 1975; Reber et al., 1979; Zeyer and Kearney, 1982). Nitrobenzene derivatives such as nitrophenols, 2,4-dinitrophenol, picric acid, nitrobenzoic acids, or 4,6-dinitro-*o*-cresol are also degraded through catechol intermediates. The nitro substituents are released as nitrite (Germanier and Wuhrmann, 1963; Gundersen and Jensen, 1955; Spain, 1979) or as ammonia (Cartwright and Cain, 1959; Tewfik and Evans, 1966; Villanueva, 1964).

In this paper, we now demonstrate the total degradation of PNA and MNA and the partial degradation of ONA by a strain of *Pseudomonas*.

MATERIALS AND METHODS

Chemicals. Chemicals and their sources included aniline hydrochloride (Aldrich Chemical Co., Milwaukee, WI), ONA and PNA (J. T. Baker Chemical Co., Phillipsburg, NJ), MNA (Matheson Coleman and Bell, Cincinnati, OH), Chloramphenicol and bovine albumin (Calbiochem-Behring Corp., La Jolla, CA), yeast extract (Difco Laboratories, Detroit, MI), [^{14}C]aniline hydrochloride (specific activity 69.0 mCi/mM; Research Products International, Elk Grove Village, IL), [^{14}C]ONA (13.0 mCi/mM) and [^{14}C]PNA (3.8 mCi/mM) (Mallinckrodt, St. Louis, MO), and [^{14}C]MNA (27.0 mCi/mM; Pathfinder Laboratories, Inc., St. Louis, MO). All radiochemicals were uniformly ring labeled and their purity was higher than 98% as indicated by the manufacturer.

Degradation of Nitroanilines in Soil. Forty grams of air-dried Matapeake silt loam [properties described by Kearney et al. (1980)] was placed in a 250-mL Erlenmeyer flask and treated with an aqueous solution (10 mL) of a ^{14}C-labeled substrate. The final concentration of each substrate in soil was 100 ppm. Some soils also received 2000 ppm of NaN_3 to stop microbial growth. The evolution of $^{14}CO_2$ was determined as indicated below.

Growth Conditions of *Pseudomonas* sp. Strain P6. Strain P6 was cultured on a basal medium supplemented with carbon and nitrogen sources as indicated under results. The composition of the basal medium was previously described (Zeyer and Kearney, 1982). All media were sterilized by filtration (0.45-μm GA-6 membrane filter, Gelman Sciences, Inc., Ann Arbor, MI). If not indicated otherwise, the pH of the media was adjusted to 7.0. All cultures (duplicate samples) were incubated on a rotary shaker under aerobic conditions at 28 °C.

Preparation of Cell Suspensions and Determination of Protein. Strain P6 was cultured on a particular medium, and the optical density at 546 nm (OD_{546}, measured on a Beckman Model 25 spectrophotometer) was determined periodically. In cultures containing PNA, the OD_{546} values were corrected for the absorbance caused by PNA. At the end of the exponential growth phase, the cells were harvested by centrifugation (Sorvall RC-2B, 4000*g*, 30 min). The sediment was washed 3 times with cold phosphate buffer (0.05 M, pH 7.0) and finally suspended in the same buffer at an OD_{546} of about 5.5, corresponding to a protein content of 1.0 mg/mL. So that enzyme synthesis could be prevented, 1 mM chloramphenicol was added to all cell suspensions. The suspensions were incubated on a rotary shaker under aerobic conditions at 28 °C with selected substrates. The protein content of all suspensions and cultures was determined by the modified method of Lowry for whole cells with bovin albumin as a standard (Herbert et al., 1971).

Determination of the Distribution of Radioactivity. The distribution of radioactivity from ^{14}C-labeled substrates in soil, cultures, or cell suspensions was determined by pulling CO_2-free air through the incubation flask and subsequently through a solution of 10 mL of 0.2 N NaOH to entrap $^{14}CO_2$. In some experiments, 10 mL of 1.0 N H_2SO_4 was placed between the incubation flask and the NaOH solution to entrap volatilized PNA. The amount of radioactivity in cultures, cell suspensions, NaOH solutions, and H_2SO_4 solutions was determined by taking 1-mL samples, adding 10 mL of Ready-Solv HP scintillation fluid (Beckman), and counting the samples in a Searle Mark II liquid scintillation counter (Searle Analytic, Inc.). Samples of 1 N H_2SO_4 were previously neutralized. The radioactivity associated with the cells was determined by filtering culture samples through a 0.45-μm GA-6 membrane filter, washing the cells on the filter with a solution of 1.0 N H_2SO_4, and combusting the filter with the cells in a Tri-Carb Model 306 sample oxidizer (Packard).

Table I. Degradation of Aniline, ONA, MNA, and PNA in Soil

^{14}C-labeled substrate added to soil[a]	$^{14}CO_2$ evolved, %			
	+NaN_3[b]		−NaN_3	
	0–10 days	10–20 days	0–10 days	10–20 days
aniline	0.6	0.2	8.5	3.8
ONA	0.2	<0.1	1.0	0.9
MNA	0.2	<0.1	1.3	2.7
PNA	0.2	<0.1	1.2	1.1

[a] The concentration in soil was 100 ppm. The radioactivity initially added was considered to be 100%. [b] The concentration in soil was 2000 ppm.

Analysis of Substrates. Samples (1 mL) of cultures or cell suspensions containing particular substrates were treated with 1 mL of methanol, shaken, and centrifuged. The supernatant was analyzed by HPLC using a Model 6000 pump equipped with a U6K injector, a Model 440 detector operating at a wavelength of 254 nm, and a Model 730 data module for the peak integration (all instruments from Waters Associates, Milford, MA). The separation was performed with a 8-mm, 5-μm Radial-PAK C18 column (Waters), using water–acetonitrile (60:40 v/v) plus 0.1% acetic acid (2 mL/min) as a solvent. The retention times of ONA, MNA, and PNA under these conditions were about 5.0, 6.2, and 3.5 min, respectively. The concentrations of ONA, MNA, and PNA in the samples were determined by injecting 20-μL samples, integrating the area of the peak at the particular retention times, and comparing the values with the peak areas of standards with known concentrations.

RESULTS

Degradation of Aniline, ONA, MNA, and PNA in Soil. The degradation of aniline and its nitrated derivatives to CO_2 in soil is shown in Table I. About 12% of the aniline was degraded to CO_2 within 20 days, which is in agreement with reported values (Suess et al., 1978). Soils supplemented with ONA, MNA, or PNA liberated only about 2–4% CO_2 within 20 days, which is in the same range as the CO_2 evolution reported for *p*-chloroaniline and 3,4-dichloroaniline (Suess et al., 1978). Less than 1% CO_2 was evolved from soils treated with NaN_3, which suggested that microorganisms contribute substantially to the ring degradation of aniline and its nitrated derivatives.

Isolation of a Microorganism Capable of Degrading PNA. Media containing 1 mM ONA, MNA, and PNA, respectively, as sole sources of carbon were inoculated with various soil samples and incubated. Samples (2 mL) of these cultures were transferred on 10 mL of fresh media every 2–4 weeks. Disappearance of the yellow color was

Table II. Degradation of PNA by Growing Cells of *Pseudomonas* sp. Strain P6:[a] Analysis of Culture after an Incubation Time of 8 Days

PNA recovered in culture, mM	<0.01
radioactivity entrapped in NaOH (=$^{14}CO_2$), %	72.7
radioactivity entrapped in H_2SO_4 (=volatilized PNA), %	<0.1
radioactivity remaining in culture (medium + cells), %	25.2
after filtration in filtrate	8.2
after filtration associated with acid-washed cells	16.3
protein content of culture, mg/mL	0.06

[a] Strain P6 was cultured on basal medium plus 1.5 mM [^{14}C]PNA plus 200 ppm of yeast extract. The radioactivity initially added was considered to be 100%.

Table III. Degradation of PNA to CO_2 by Growing Cells of *Pseudomonas* sp. Strain P6 at Different Medium pHs[a]

initial pH of medium	$^{14}CO_2$ evolved within 8 days, %
5.0	10
6.0	76
6.5	76
7.0	72
7.5[b]	75
8.0[b]	61
8.5[b]	32

[a] Strain P6 was cultured on basal medium plus 1.0 mM [^{14}C]PNA plus 200 ppm of yeast extract. [b] The cultures were acidified previous to the determination of $^{14}CO_2$.

Table IV. Degradation of PNA to CO_2 by Growing Cells of *Pseudomonas* sp. Strain P6 at Different Concentrations of PNA[a]

concn of [^{14}C]PNA, mM	$^{14}CO_2$ evolved within 8 days, %
1.0	72
1.5	74
2.0	58
2.5	54
3.0	44
3.5	9
4.0	<2

[a] Strain P6 was cultured on basal medium plus [^{14}C]-PNA plus 200 ppm of yeast extract.

taken as an indicator of degradation.

All media containing ONA or MNA remained yellow, even after incubation times of several months. A culture enriched for 10 weeks, on the media containing PNA, however, decolorized the medium. From this culture, a bacterial strain capable of degrading PNA was isolated. Cultures of this bacterium consisted of small Gram-negative rods. The strain was cytochrome and catalase positive and was identified according to "Bergey's Manual of Determinative Bacteriology" (Breed et al., 1957) and coded as *Pseudomonas* sp. strain P6.

Several other approaches to enrich microbial cultures on ONA, MNA, or PNA failed. Soil perfusion systems (Kaufman, 1966) and chemostats (Harder et al., 1977) were inoculated with various soil samples and incubated with nitroanilines as substrates over several months, but we were unable to observe any significant enrichment of microorganisms. Degradation of ONA, MNA, and PNA in these enrichment systems was periodically assayed by using ^{14}C-labeled compounds, but $^{14}CO_2$ evolution was always found to be below 0.5%/day.

In another approach, cultures of microorganisms were enriched on analogues of nitroanilines. We isolated pure cultures growing on aniline, *m*-chloroaniline, *p*-chloroaniline (Zeyer and Kearney, 1982), *o*-, *m*-, and *p*-nitrophenol, *p*-aminobenzoic acid, *p*-nitrobenzoic acid, or anthranilic acid as the sole source of carbon. Some of these organisms were able to reduce nitroanilines to the corresponding diamino derivatives in the presence of particular analogues. However, no significant degradation of ONA, MNA, or PNA to CO_2 was observed. In similar experiments reported by Malaney (1960) and Chambers et al. (1963), no significant nitroaniline degradation occurred in aniline- or phenol-adapted cultures.

Degradation of ONA, MNA, and PNA by Growing Cells of *Pseudomonas* sp. Strain P6. Strain P6 grew slowly on basal medium plus 1 mM [^{14}C]PNA as a sole source of carbon. The culture released 60–80% of the initial radioactivity as $^{14}CO_2$ within about 1 month, and no PNA was found in the culture (determined by HPLC) at the end of the incubation. The incubation time, however, was too long to allow a detailed analysis of the degradation products. Several factors (evaporation of medium, contaminations, nonenzymatic reactions) diminished the accuracy of balance studies (data not shown). The growth rate was enhanced considerably by the addition of yeast extract. Strain P6, growing on 1.5 mM [^{14}C]PNA plus 200 ppm of yeast extract, degraded PNA completely within 8 days (Table II). Of the initial radioactivity, 73% was evolved as $^{14}CO_2$, 16% was associated with the cells, and 8% was found to be in the culture filtrate. The yield of $^{14}CO_2$ did not increase by acidifying the culture or by extending the incubation time.

The degradation of aniline, *p*-chloroaniline, and *p*-nitrophenol was reported to depend on the pH of the medium (pH optima: 6.5, 6.0, and 7.8) (Gundersen and Jensen, 1955; Helm and Reber, 1979; Zeyer and Kearney,

Table V. Degradation of ONA, MNA, and PNA by Growing Cells of *Pseudomonas* sp. Strain P6

substrates added to basal medium plus 200 ppm of yeast extract[a]	analysis of cultures: $^{14}CO_2$ evolution, % 0–7 days	$^{14}CO_2$ evolution, % 0–16 days	radioact in culture after 16 days, %	substrate concn in culture after 16 days, mM
1 substrate				
[^{14}C]ONA	<1	<1	98	>0.86
[^{14}C]MNA	<1	<1	102	>0.90
[^{14}C]PNA	71	76	26	<0.01
2 substrates				
[^{14}C]ONA + PNA	<1	<1	102	ONA, 0.49; PNA, <0.01
[^{14}C]MNA + PNA	14	33	69	MNA, <0.01; PNA, <0.01
ONA + [^{14}C]PNA	72	75	20	ONA, 0.53; PNA, <0.01
MNA + [^{14}C]PNA	35	65	38	MNA, <0.01; PNA, <0.01

[a] The initial concentration of each substrate was 1.0 mM. The radioactivity initially added was considered to be 100%. All media (10 mL) were inoculated with 0.4 mL of a culture of strain P6 pregrown on basal medium plus 1.5 mM PNA plus 500 ppm of yeast extract.

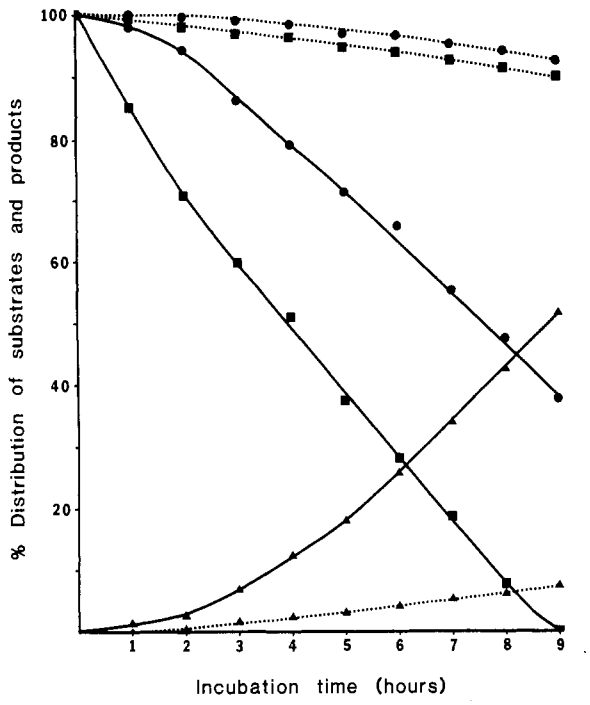

Figure 1. Degradation of MNA and PNA by resting cells of *Pseudomonas* sp. strain P6 precultured in the presence of PNA. Strain P6 was precultured on basal medium plus 2 mM PNA plus 500 ppm of yeast extract and suspended in buffer. The cell suspension was divided into two volumes, which were incubated with [^{14}C]MNA or [^{14}C]PNA. The initially added concentrations of substrate and radioactivity were considered to be 100%. The protein content of the suspensions was 1.0 mg/mL. (---) Cell suspension incubated with 1 mM [^{14}C]MNA; (—) cell suspension incubated with 1 mM [^{14}C]PNA; (●) total radioactivity in suspension; (■) [^{14}C]substrate in suspension; (▲) evolution of $^{14}CO_2$ from suspension.

1982). In contrast, the degradation of PNA to CO_2 by strain P6 was relatively independent of the pH of the medium within the range pH 6–8 (Table III). At pH 7, up to 3 mM PNA were rapidly degraded (Table IV).

Degradation of isomers of PNA by strain P6 is shown in Table V. Although no significant degradation of ONA and MNA was found in absence of PNA, about 50% ONA and 100% MNA were metabolized when PNA was present. ONA was degraded to nonvolatile products which remained in the culture. Attempts to identify these products by HPLC failed. MNA, however, was metabolized to CO_2 in the presence of PNA. The rate of the degradation of 1 mM PNA to CO_2 by strain P6 was not affected by the presence of 1 mM ONA but was slightly reduced in the presence of 1 mM MNA, which may indicate a competitive inhibition of a permease or a catabolic enzyme by MNA.

Degradation of MNA and PNA by Resting Cells of *Pseudomonas* sp. Strain P6. Cells of strain P6 were precultured on basal medium plus 2 mM PNA plus 500 ppm of yeast extract, harvested, and suspended in buffer. The cell suspension was incubated with 1 mM [^{14}C]MNA or [^{14}C]PNA. Decrease of the ^{14}C-labeled substrate, loss of radioactivity from the suspensions, and evolution of $^{14}CO_2$ are shown in Figure 1. The suspended cells were able to degrade MNA and PNA to CO_2. The activity of the enzymes involved in the metabolism was higher on PNA than on MNA which is in agreement with the data reported in Table V. Taking the rate of the PNA degradation and the protein content of the suspension into consideration, the specific degradation rate was calculated to be about 2 μmol of PNA min^{-1} (g of protein)$^{-1}$. The rate of the MNA degradation was only 10% of this value. Resting cells of strain P6 pregrown on basal medium plus 500 ppm of yeast extract were unable to degrade MNA and PNA (data not shown). This indicated that the enzymes involved in the metabolism of MNA and PNA are not constitutive but inducible by PNA.

Figure 1 shows a fast initial degradation of [^{14}C]PNA but only a slow evolution of $^{14}CO_2$. This fact suggested a temporary accumulation of nonvolatile intermediates. Samples were taken hourly from the suspension and analyzed by HPLC for possible metabolites. By use of a Radial-PAK C18 column and an appropriate mixture of methanol–water (plus 0.1% acetic acid or 0.01 M ammonium phosphate) as a solvent, standards of 4-nitrocatechol, 1,2,4-benzenetriol, *p*-hydroquinone, *p*-nitrophenol, *p*-hydroxyaniline, and *p*-phenylenediamine showed particular peaks. No significant concentrations of these possible metabolites, however, were found in the cell suspension.

DISCUSSION

Pseudomonas sp. strain P6 grew slowly on 1 mM PNA as a sole source of carbon. The degradation of PNA was much faster in the presence of 200 ppm of yeast extract, presumably because it provided the organism with growth factors. Resting cells precultured on yeast extract alone exhibited no activity on PNA, and therefore it is unlikely that yeast extract enhances the specific degradation activity of growing cells.

Bringmann and Kuehn (1980) studied the toxicity of PNA to several organisms (*Pseudomonas*, green algae, protozoa) active in the biological self-purification of river water. Growth of each organism was significantly inhibited in the presence of 0.1 mM PNA. Young and Affleck (1974), working with sewage sludge adapted to PNA, found a significant reduction of the BOD at about 2 mM PNA. We have no data on growth inhibition of strain P6 by PNA, but concentrations up to 3 mM PNA were degraded rapidly to CO_2. Metabolism of 1 mM PNA also occurred in presence of 1 mM ONA or MNA. Strain P6 seems to be rather tolerant of high concentrations of nitroanilines.

MNA was not degradable by strain P6 as a sole source of carbon; PNA, however, was able to induce the enzymes involved in the metabolism of MNA and PNA. Young and Affleck (1974) made the same observation while studying MNA and PNA degradation in sewage sludge (see the introduction). This suggests a similar metabolic pathway for MNA and PNA. A degradation of MNA and PNA through a common intermediate may be possible; however, attempts to isolate and identify any intermediates by HPLC failed. Reber et al. (1979) postulated a similar metabolic pathway for *m*-chloroaniline and *p*-chloroaniline. Initial oxidation of both substrates by a *Pseudomonas* sp. led to a temporary accumulatin of 4-chlorocatechol which, in turn, was subject to ring cleavage.

Registry No. PNA, 100-01-6; ONA, 88-74-4; MNA, 99-09-2.

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Concentration, Insulin Potentiation, and Absorption of Chromium in Beer

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The chromium content of 27 domestic and imported brands of beer was determined to evaluate the importance of beer as a dietary source of chromium. Chromium concentration of selected beers ranged from 0.48 to 56 ng/mL; concentration of 8 brands of beer was below 2 ng/mL, 11 ranged from 2 to 10, and 8 brands of beer contained more than 10 ng of Cr/mL. Chromium content of the ingredients was not sufficient to account for the high levels of Cr found in some beers; therefore, a significant amount of Cr in beer was due to unidentified sources. Chromium in beer, brewing water, and corn grits displayed insignificant in vitro insulin potentiating activity, malt displayed low levels of insulin activity, and hops, which was the highest in total Cr, inhibited in vitro insulin activity. Chromium in beer was absorbed by humans similar to the Cr found endogenously in foods. These data demonstrate that the Cr concentration of beer varied widely and that some beer contained significant quantities of chromium that was absorbed by humans.

Marginal chromium deficiency of man and animals leads to impaired glucose and lipid metabolism that can be prevented or alleviated by proper chromium nutrition (Schwarz and Mertz, 1959; Jeejeebhoy et al., 1977). Even individuals who eat a well-balanced diet may not be ingesting the suggested minimum recommended safe and adequate amount of chromium (Kumpulainen et al., 1979). Brewers' yeast is one of the richest sources of chromium but relatively few people normally eat brewers' yeast. Many people, however, frequently drink beer, the end product of the brewers' yeast fermentation process. The average per capita consumption of beer in the United States is approximately 9 oz/day compared to 8.6 oz for milk (Katz, 1981).

Among the constituents of beer, several metals are nutritionally important, including sodium, potassium, calcium, magnesium, and trace quantities of copper, iron, and zinc (Binns et al., 1978). Chromium should also be added to that list. In the present study, significant quantities of Cr were present in several brands of beer; on the basis of the average Cr content of the 27 brands of beer analyzed in this study, an average 12-oz (355 mL) beer would supply 7% of the minimum recommended safe and adequate intake for Cr. Relative absorption, sources of Cr, and forms of Cr in beer and ingredients were also determined.

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MATERIALS AND METHODS

Beer samples were purchased from local liquor stores or donated by individuals working at the Beltsville Human Nutrition Research Center. Ingredients used in brewing beer were provided by Carling National Brewery, Baltimore, MD, and Stroh Brewery, Detroit, MI. Insulin potentiation of samples was determined as described (Anderson et al., 1978a). Beer samples (10 brands) were assayed directly; beer ingredients were extracted with 0.1 N ammonium hydroxide (Anderson et al., 1978b) and analyzed in duplicate on 2 separate days for insulin potentiating activity.

Chromium Analysis of Urine and Beer. Chromium was determined with flameless atomic absorption spectroscopy by method of additions on nonashed urine samples (25 μL) with a Perkin-Elmer 5000 and a HGA-500 furnace with pyrolytic coated tubes. Furnace conditions for direct analysis of Cr in urine were the following: first drying, 100 °C; ramp, 15 s; hold, 20 s; internal argon drying flow, 300 mL/min; second drying, 130 °C; ramp, 15 s; hold, 60 s; internal argon flow, 300 mL/min; atomize, 2700 °C; ramp, 0 s; hold, 4 s; internal argon flow, 50 mL/min; clean out, 2700 °C; ramp, 1 s; hold, 4 s; internal argon flow, 300 mL/min. Urine samples were collected in plastic-lined