Biochemical Pharmacology, Vol. 31, No. 2, pp. 239–247, 1982.
Printed in Great Britain.

0006-2952/82/020239-09 $03.00/0

MIXED FUNCTION OXIDASE IN THE MAMMARY GLAND AND LIVER MICROSOMES OF LACTATING RATS

EFFECTS OF 3-METHYLCHOLANTHRENE AND β-NAPHTHOFLAVONE*†

CLARE L. RITTER and DANUTA MALEJKA-GIGANTI‡
Laboratory for Cancer Research, Veterans Administration Medical Center, and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55417, U.S.A.

(*Received* 15 *April* 1981; *accepted* 13 *June* 1981)

Abstract—Mammary gland and liver microsomes of lactating rats were examined for the components of mixed function oxidase and related enzyme activities. Cytochrome b_5, NADH- and NADPH-dependent cytochrome *c* reductase activities were 15-, 6- and 10-fold lower, respectively, in the mammary gland than in the liver microsomes. The determination of cytochrome P-450 (P-448) in the mammary gland microsomes required elimination of the spectral interferences by hemoglobin and cytochrome aa_3. The presence of the latter in this fraction was also shown by cytochrome *c* oxidase activity. Cytochrome aa_3 was reduced by anaerobic incubation of mammary gland microsomes, in the presence of antimycin A, with sodium succinate, phenazine ethosulfate, and sodium ascorbate for 30 min at room temperature. Spectral resolution of the dithionite-reduced cytochrome P-450 (P-448) carbon monoxide complex occurred 30 min after gassing. The basal level of cytochrome P-450 was about 500-fold greater in the liver than in the mammary gland microsomes. Pretreatment of lactating rats with the inducers of hepatic cytochrome P-448, 3-methylcholanthrene and β-naphthoflavone, increased the cytochrome content 3- to 10-fold and 2-fold, in the mammary gland and liver microsomes, respectively. The induction of cytochrome P-448 in microsomes of both tissues was also shown by type I binding spectra obtained with *N*-2-fluorenylacetamide. Using hydroxylation of benzo[*a*]pyrene and *N*-2-fluorenylacetamide as a measure of mixed function oxidase activity, we found that the basal activities, which were 4- to 8-fold greater in the liver microsomes, were increased in both tissues after treatment of rats with the inducers. The induced activities were inhibited by 0.1 mM α-napthoflavone *in vitro*, indicating a dependence on cytochrome P-448. The data suggest that the mammary gland, an extrahepatic target for carcinogens, is capable of their metabolism.

Several investigators have reported the induction of aryl hydrocarbon hydroxylase activity in the rat mammary gland with the inducers of the hepatic enzyme [1–5]. Hence, it has been assumed than an enzyme system similar to that of the hepatic mixed function oxidase metabolizes xenobiotics in the mammary gland. A component of this enzyme system, NADPH-dependent cytochrome *c* reductase, had been found and characterized in the mammary gland microsomes of the rat along with other redox constituents [6, 7]. However, cytochrome P-450(P-448), a terminal catalyst of the oxidation reactions, could not be detected in the mammary gland microsomes with the procedures routinely used for the assay of hepatic cytochrome P-450(P-448) [7, 8].

Since the content of cytochrome P-450 is generally low in extrahepatic tissues [9], we directed our efforts at spectral resolution of concentrated suspensions of mammary gland microsomes and establishment of suitable conditions for detection of the reduced cytochrome P-450(P-448) carbon monoxide complex. By eliminating interference from hemoglobin and the mitochondrial contaminant, cytochrome aa_3, we showed cytochrome P-450 and its 3-methylcholanthrene- (3-MC)§ or β-NF-inducible form, cytochrome P-448, in the mammary gland microsomes of lactating rats. We also found that mammary gland and liver microsomes of 3-MC- or β-NF-treated rats had increased cytochrome P-448-dependent capacities for hydroxylation of the carcinogens, BP and 2-FAA.

MATERIALS AND METHODS

Labeled and unlabeled compounds. BP and 2-FAA were purchased from the Aldrich Chemical Co., Milwaukee, WI. BP, m.p. 182–183°, was mixed with [7,10-^{14}C]BP (16 mCi/mmole) (California Bionuclear Corp., Sun Valley, CA) to obtain the desired activity of the labeled substrate for the enzyme assays. 2-FAA was recrystallized from ethanol–water (7:3), had a m.p. of 196–198° and was chromatographically pure [10]. 2-[1′-^{14}C]FAA, m.p. 194–196°, specific radioactivity of 1.30 mCi/mmole, was prepared as described previously [10]. 3-MC, m.p. 181–182°, was

* This investigation was supported by PHS Grant CA-28000 awarded by the National Cancer Institute, DHHS, and by the U. S. Veterans Administration.

† Presented in part at the 72nd Annual Meeting of the American Association for Cancer Research at Washington, DC, April 27–30, 1981.

‡ Author to whom all correspondence should be addressed: Danuta Malejka-Giganti, Laboratory for Cancer Research, Veterans Administration Medical Center, Building 31, 151B, 54th St. & 48th Ave. S., Minneapolis, MN 55417, U.S.A.

§ Abbreviations: 3-MC, 3-methylcholanthrene; β-NF, naphthoflavone, or 5,6-benzoflavone; BP, benzo[*a*]pyrene; 2-FAA, *N*-2-fluorenylacetamide; α-NF, naphthoflavone, or 7,8-benzoflavone; and DTT, dithiothreitol.

purchased from the Eastman Kodak Co., Rochester, NY. α-NF and β-NF, the sodium salts of ascorbic and succinic acids, antimycin A (type III), phenazine ethosulfate, cytochrome *c* from horse heart (type III), NADH, NADPH and dithionite were purchased from the Sigma Chemical Co., St. Louis, MO.

Animals, maintenance and treatment. Sprague–Dawley rats (Holtzman Co., Madison, WI), 4–6 months old, were maintained on regular Purina Chow Pellets and water *ad lib*. The rats were mated and pregnant dams were transferred to individual breeding boxes. Those with ten to fourteen offspring were selected at different stages of lactation. The lactating rats were injected twice or thrice i.p. with 3-MC or β-NF disolved in corn oil at concentrations of 8 or 16 mg/ml to achieve dose levels of 20 or 40 mg/kg of body weight. Food was withdrawn 12 hr before decapitation of rats.

Preparation of microsomal and mitochondrial fractions. All buffers were cooled in ice and all procedures were carried out at 4° except where noted. The mammary gland was excised at room temperature and immersed in cold 154 mM KCl, 50 mM Tris–HCl buffer, pH 7.4. The gland was cleaned of muscle and lymph nodes and was minced in a small volume of the above buffer. The mince was washed thrice with the same buffer to remove hemoglobin and filtered through 0.2 mm nylon mesh. The mince was then suspended in three parts (w/w) of the same buffer and homogenized with a Polytron homogenizer (type PT 10, Kinematica GMBH, Lucerne, Switzerland) for three intervals of 3 sec each at a speed setting of 3.0. The homogenate was centrifuged at 600 *g* for 10 min and the pellet was resuspended in a small volume of the same buffer and homogenized for an additional 3 sec. The combined supernatant fractions were then centrifuged at 16,000 *g* for 20 min to obtain the mitochondria. The supernatant fraction was centrifuged at 105,000 *g* for 60 min to separate the microsomes. To further remove hemoglobin, both mitochondrial and microsomal fractions were suspended in 1 mM Tris–HCl buffer, pH 7.4, and homogenized gently in a Teflon pestle homogenizer [11]. The suspensions were centrifuged at 105,000 *g* for 60 min. The washed pellets were then suspended in 10 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM DTT buffer, pH 7.5, containing 20% glycerol to a protein concentration of 30 mg/ml. The liver was perfused with 154 mM KCl, 50 mM Tris–HCl buffer, pH 7.4, excised, and immersed in the same buffer. After removal of connective tissue, the liver was minced in four parts (w/w) of the same buffer and homogenized as above. The liver homogenate was treated identically to the mammary gland homogenate except that the homogenization of the pellet from the 600 *g* centrifugation was omitted. For electron microscopy, small portions of the washed microsomal pellets from the tissues of representative rats were fixed and processed as described previously [12].

Determination of protein and cytochromes b_5, aa_3 *and P-450(P-448).* Protein was determined by the procedure of Lowry *et al.* [13] with bovine serum albumin as a standard. For spectrophotomeric determinations of cytochromes, the mitochondrial and microsomal suspensions were diluted with 300 mM sodium phosphate buffer, pH 7.4, containing 50% glycerol to protein concentrations of 1 mg/ml and 6–10 mg/ml for liver and mammary gland preparations respectively. Cytochrome levels were measured at room temperature. Cytochrome b_5 was determined from the NADH-reduced spectrum (ε, 185 $mM^{-1}\,cm^{-1}$) [14]. The content of cytochrome aa_3 was estimated from the 605 minus 630 nm absorbances of the dithionite-reduced versus oxidized spectrum (ε, 13.1 $mM^{-1}\,cm^{-1}$) [15]. Anaerobic conditions were maintained in the reduced sample in a septum-covered cuvette by bubbling nitrogen treated with Oxiclear (Pierce Chemical Co., Rockford, IL) for 2 min. Cytochrome P-450(P-448) in the liver was determined from the reduced hemoprotein–carbon monoxide complex (ε, 91 $mM^{-1}\,cm^{-1}$) [14].

The determination of cytochrome P-450(P-448) in the mammary gland was modified as follows: to 1.2 ml of the membrane preparation in the reference cuvette were added 10 μl of 0.6 mM antimycin A in 95% ethanol and 20 μl each of 370 mM sodium succinate, 0.37 mM phenazine ethosulfate, and 37.5 mM sodium ascorbate. The same additions were made to the sample except that the antimycin A was replaced by 10 μl of 95% ethanol. Oxygen-free nitrogen was then bubbled for 2 min through both septum-covered cuvettes, which were then allowed to stand for 30 min. After the initial 20 min, a few crystals of dithionite were added to the sample cuvette, followed by bubbling with nitrogen for 2 min. Carbon monoxide (Matheson, research grade) was then gently bubbled through both cuvettes for 2 min and the spectra were recorded 10, 30 and 45 min after bubbling. The content of cytochrome P-450(P-448) was calculated from the difference in absorbances at the maximum of 450–448 nm and the minimum of 465–462 nm with the use of an extinction coefficient of 104 $mM^{-1}\,cm^{-1}$ [16]. Total heme content was determined by the method of Paul *et al.* [17]. The spectra of mammary gland and liver preparations were recorded with a Varian Cary 219 spectrophotometer with automatic baseline correction and a Beckman Acta VI spectrophotometer fitted with a scattered transmission accessory respectively.

Assays of reductase and cytochrome c *oxidase activities.* The rates of cytochrome *c* reduction were determined at room temperature essentially as described [18], using 300 mM potassium phosphate buffer, pH 7.5, containing 0.10 mM EDTA, 0.07 mM cytochrome *c* and 0.10 mM NADPH or 0.12 mM NADH in a 1.0 ml volume. NADH–ferricyanide reductase activity was measured as described [19], except that 50 mM potassium phosphate buffer, pH 7.5, 0.43 mM potassium ferricyanide and 0.24 mM NADH were used. NADH- and NADPH–cytochrome *c* reductase activities were measured in the absence and presence of 1 mM potassium cyanide. The NADH–cytochrome *c* and NADH–ferricyanide reductase activities were also measured with and without 0.01% rotenone. Cytochrome *c* oxidase activity was determined spectrophotometrically as previously described [20]. The cytochrome *c* was reduced immediately before use with a small amount of dithionite. Excess dithionite was removed by bub-

bling air through the solution for 2–3 min. To prevent oxidation of the reduced cytochrome *c*, the solution was then bubbled with oxygen-free nitrogen and kept in a septum-covered vial for a maximum of 30 min.

Determination of binding of 2-FAA *to mammary gland and liver microsomes.* Microsomes from tissues of 3-MC- or β-NF-treated lactating rats were suspended in 300 mM sodium phosphate buffer, pH 7.5, containing 50% glycerol, at a concentration of 8.0 or 4.0 mg protein/ml for the mammary gland or liver respectively. 2-FAA in propylene glycol (20 μl) was added at a concentration of 0.12 mM to the sample cuvette containing 1.2 ml of the microsomal suspension. The reference cuvette contained only the microsomes in 1.2 ml buffer and propylene glycol (20 μl). The mixtures were allowed to stand at room temperature for 10–30 min before the spectra were recorded from 450 to 380 nm. A Hitachi 110 computerized double beam ratio recording spectrophotometer was used.

Determination of hydroxylation of BP. A modified radioactive assay [21] for measurement of BP hydroxylation was employed. The microsomes, 1.2 mg protein, suspended in 0.95 ml of 100 mM potassium phosphate buffer, pH 7.2, were placed in scintillation vials. The suspensions were preincubated with 0.10 mM α-NF in 10 μl of 95% ethanol or with the solvent alone for 5 min at 37° before the additions of 40 nmoles [7,10-^{14}C]BP, containing approximately 100,000 dpm, in 30 μl of acetone and of 0.84 mM NADPH in 50 μl of the buffer. Incubations to measure microsomal activities of liver and mammary gland were carried out for 10 and 30 min, respectively, in a Dubnoff metabolic incubator at 37° in darkness in vials open to air. The control incubations contained microsomes that had been immersed in a boiling water bath for 5 min. All incubations were carried out at least in duplicate. The assays were performed under conditions of linearity with respect to time and protein concentration. The reactions were stopped by addition of 1 ml of acetone, followed immediately by 3 ml of hexane. The vials were then capped, shaken for 10 min at 37° in the incubator, subjected to vigorous mixing with a Vortex mixer for 20 sec, and placed in an ice-bath. The hexane layer was aspirated into a new set of vials and extracted with 2 ml of 1 N NaOH by rapid mixing with a Vortex mixer. The hexane layer was again removed by aspiration and the aqueous phase containing the phenolic metabolites of [7, 10-^{14}C]BP was mixed with the scintillator described previously [22].

Determination of hydroxylation of 2-*FAA.* The composition of the incubation mixtures was the same as above except that the buffer (pH 7.4) and the substrate 2-[1′-^{14}C]FAA were used. The reactions were stopped by addition of 1 ml of cold 1 M sodium acetate buffer, pH 6.0. The mixtures were extracted twice with 3 ml of ethyl ether by rapid mixing with a Vortex mixer. The ether phases were aspirated, combined, and extracted once with 2 ml of 0.1 N NaOH again by vigorous mixing. After removing the ether phase, the aqueous phase containing the phenolic and *N*-hydroxy metabolites of 2-[1′-^{14}C]FAA was mixed with the scintillator described previously [22].

Radioactivity measurements. Radioactivity was determined using a Packard Tri-Carb Liquid Scintillation Spectrometer, model 3255. The counts were corrected for quenching by means of an external standard. The counting efficiency was 68–73%.

RESULTS

Reduction of cytochrome P-450(P-448) in the microsomes is a prerequisite for its complexation with carbon monoxide [14]. When spectra of the reduced microsomes from mammary gland were compared to those from the liver, marked differences were noted. Reduction of the hepatic microsomes with NADH or dithionite gave nearly identical spectra with absorbance maxima at 555 and 424 nm, which indicated the presence of cytochrome b_5. In the dithionite-reduced mammary gland microsomes, maxima were located at 607–603, 558–555, 530, 443 and 430–428 nm, whereas in the NADH-reduced microsomes, the maxima were located at 560 (shoulder), 554 and 426–424 nm. The absorbances

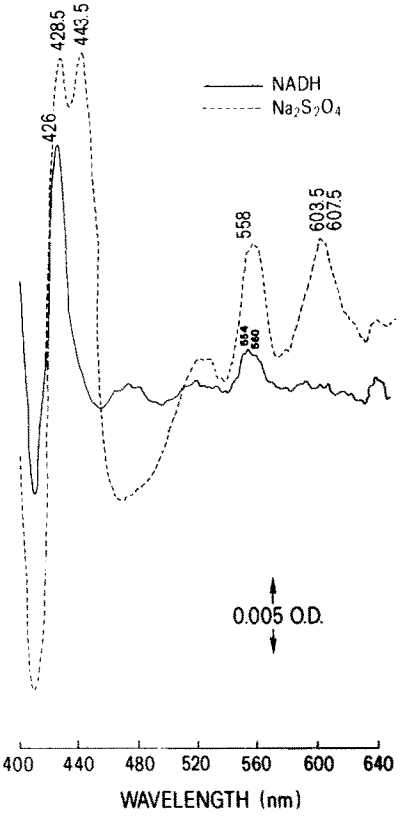

Fig. 1. Reduced versus oxidized difference spectra of the mammary gland microsomes of a lactating rat. Microsomes suspended in 300 mM sodium phosphate buffer, pH 7.5, containing 50% glycerol (4.0 mg protein/ml) were divided between two cuvettes (1.2 ml each) and the baseline was recorded. To the sample cuvette, which was kept anaerobic, were added a few grains of NADH, followed by bubbling with oxygen-free nitrogen for 2 min (——). Then a few grains of dithionite were added to the same cuvette, followed by bubbling with nitrogen for 2 min (– – – –). Throughout, the reference cuvette was open to air. A Varian Cary 219 spectrophotometer with automatic baseline correction was used (1.8 nm band width).

corresponding to cytochrome b_5 (426–424 nm) and to the α band of the b-cytochrome (555 nm) were considerably less with NADH used as the reducing agent than with dithionite. Saturation with carbon monoxide of the dithionite-reduced mammary gland microsomes shifted the 443 nm peak to 430 nm, and saturation of the unreduced microsomes produced a peak at 420 nm. This suggested that our preparation of mammary gland microsomes was contaminated by hemoglobin and cytochrome aa_3. Because of the generally low content of cytochrome P-450(P-448) in microsomes of extrahepatic tissues [9] and small amounts of cytochrome b_5 in mammary gland [7], the effects of the above contaminants on measurements of the cytochromes could be significant and had to be eliminated.

Hemoglobin which is reduced by dithionite has absorbance maxima at 555 and 430 nm, and in the unreduced state forms a carbon monoxide complex with an absorbance maximum at 420–418 nm [23]. To minimize contamination of mammary gland microsomes by hemoglobin, the minced tissue was washed with a buffer containing 154 mM KCl and the microsomal pellet was washed once with a hypotonic buffer, 1 mM Tris–HCl (pH 7.4), a procedure found effective for removal of hemoglobin in lung microsomes [11]. Additional washings did not further decrease the contamination. The effectiveness of these washings in minimizing contamination of mammary gland microsomes with hemoglobin was determined by the relative absorbance at 420 nm of the carbon monoxide-saturated microsomes. The removal of hemoglobin resulted in a decrease in the absorbances at 555 and 430 nm and a shift of the 430 nm peak to 428–426 nm in the dithionite-reduced microsomes (Fig. 1). The absorption peaks at 603 and 443 nm in the dithionite-reduced spectrum of the mammary gland microsomes and the shift in the 443 nm peak to 430 nm after gassing the reduced sample with carbon monoxide were ascribed to cytochrome aa_3 [24]. The content of cytochrome aa_3 was estimated from the reduced spectrum to be about 100 pmoles per mg of protein. Using the conventional method of cytochrome P-450(P448) determination [14], a trough appeared in the spectrum at 443 nm from the dithionite-reduced cytochrome aa_3 in the reference which was not compensated for in the carbon monoxide-treated sample. Thus, even slight contamination by cytochrome aa_3 could drastically affect the spectral determination of low levels of cytochrome P-450(P-448).

The extent of contamination of the microsomal fraction by the mitochondrial enzyme can be estimated from cytochrome c oxidase activity. The measurements indicated that mitochondrial enzyme activity was greater in the mammary gland than in the liver microsomal fraction (Table 1). Attempts to separate mitochondria through two consecutive centrifugations of the 600 g supernatant fraction, at 10,000 g for 10 min and at 16,000 g for 20 min, did little to decrease the mitochondrial enzyme contamination of the mammary gland microsomal fraction. Since the electron micrograph of the mammary gland microsomal fraction showed no mitochondrial structures, we assumed that mitochondrial enzymes were released during the homogenization. Omission of the additional homogenization of the mammary gland 600 g pellet did not decrease cytochrome c oxidase activity in the microsomal fraction. Consequently, our efforts were directed toward eliminating the spectral interference by cytochrome aa_3. Our aim was to selectively reduce the cytochrome aa_3 while maintaining cytochrome P-450 in its unreduced state. Since antimycin A, a mitochondrial electron transport uncoupling agent, has been reported to prevent the backflow of electrons from ascorbate to NADH and cytochrome P-450 [25], it was added to the reference cuvette before the reducing agents. However, no differences were detected in measurements taken in its absence. The reduction of cytochrome aa_3 was accomplished by anaerobic incubation with 6.0 mM sodium succinate, 0.006 mM phenazine

Table 1. Cytochrome c oxidase activity in the mammary gland and liver of lactating rats

	Cytochrome c oxidase activity (nmoles · min^{-1} · mg^{-1})†					
	Mammary gland			Liver		
Pretreatment of rat*	Microsomal fraction	Mitochondrial fraction	Ratio‡	Microsomal fraction	Mitochondrial fraction	Ratio‡
Corn oil§	91 ± 57	250 ± 42	0.36	61 ± 4	387 ± 57	0.16
Corn oil‖	189 ± 68	667 ± 100	0.28	44 ± 10	435 ± 244	0.10
3-MC§	126 ± 22	261 ± 102	0.48	67 ± 24	450 ± 179	0.15
3-MC‖	305 ± 91	895 ± 57	0.34	40 ± 10	1123 ± 291	0.04
β-NF§	147 ± 48	304 ± 135	0.48			
β-NF‖	177 ± 43	638 ± 306	0.28	32 ± 20	640 ± 175	0.05
β-NF¶	101 ± 27	290 ± 216	0.35	13 ± 4	346 ± 113	0.04

* Rats received i.p. injections of the vehicle (corn oil) or of the compound (20 or 40 mg/kg of body weight) for 3 consecutive days.

† Values are the means ± S.D. from assays on the fractions of three to seven rats.

‡ The ratio of the cytochrome oxidase activities of the microsomal and mitochondrial fractions.

§ The microsomal and mitochondrial pellets were washed by resuspension in 10 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM DTT buffer, pH 7.5.

‖ The microsomal and mitochondrial pellets were washed by resuspension in 1 mM Tris–HCl buffer, pH 7.4, as described in Materials and Methods.

¶ Mitochondria were removed by consecutive centrifugations at 10,000 g for 10 min and at 16,000 g for 20 min; the mitochondrial pellets were combined and washed as described in the ‖ footnote.

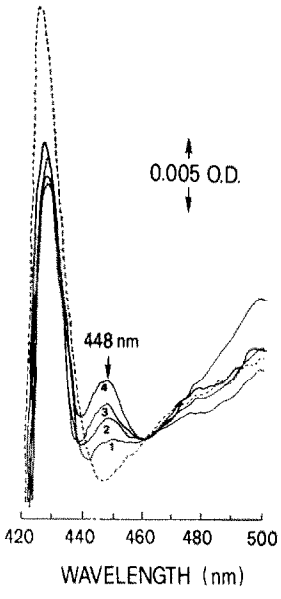

Fig. 2. Carbon monoxide difference spectra of the reduced mammary gland microsomes from a β-NF-treated lactating rat. Microsomes in 300 mM sodium phosphate buffer, pH 7.5, containing 50% glycerol (6.0 mg protein/ml) were divided between two cuvettes (1.2 ml each) and the baseline was recorded. To the reference cuvette were added 10 μl 0.6 mM antimycin A in 95% ethanol and 20 μl each of 370 mM sodium succinate, 0.37 mM phenazine ethosulfate and 37.5 mM sodium ascorbate, followed by bubbling with oxygen-free nitrogen for 2 min. The mixture was allowed to stand at room temperature for 30 min. To the sample cuvette all the reagents above except antimycin A were added and dithionite was added after the initial 20 min. The dithionite-reduced spectrum (------) was then recorded. Oxygen-free carbon monoxide was then bubbled through both cuvettes for 2 min. Spectra were recorded after 10 min (——[1]), 25 min (——[2]), 40 min (——[3]) and 70 min (——[4]). A Varian Cary 219 spectrophotometer with automatic baseline correction was used (1.97 nm band width).

ethosulfate and 0.6 mM sodium ascorbate at room temperature for 30 min. When both the sample and reference were reduced under these conditions, incubation of the sample with dithionite for 10 min produced no change in the baseline at 443 nm, indicating complete reduction of cytochrome aa_3 by the above reagents. In many spectral determinations, the absorbance of the dithionite-reduced sample was noted to decrease gradually from 500 to 460 nm (Fig. 2). This phenomenon has been reported by other investigators and has been attributed to the reduction of flavoprotein and non-heme iron protein [6, 26]. When carbon monoxide was bubbled through both the reference and sample, the effect of the reduced carbon monoxide cytochrome aa_3 complex would be balanced in both cuvettes, whereas only the dithionite-reduced cytochrome P-450(P-448) in the sample could form a carbon monoxide complex absorbing at 450 (448) nm. We observed that the complexation of the reduced cytochrome P-450 (P-448) in the mammary gland microsomes with carbon monoxide improved with time (Fig. 2). An acceptable resolution of the 450 (448) nm peak was usually obtained about 30 min after gassing with carbon monoxide. A distinct peak at 448 nm was obtained with mammary gland microsomes from 3-MC- or β-NF-treated rats, whereas often only a shoulder at 450 nm was recorded with microsomes from control (corn oil-treated) rats. The possibility of partial reduction of cytochrome P-450(P-448) by the reagents used to reduce cytochrome oxidase, in spite of the presence of antimycin A in the reference cuvette, was considered. However, no reduction of hepatic cytochrome P-450(P-448) took place under the same conditions and, after reaching a maximum value, the levels of cytochrome P-450(P-448) in the mammary gland remained fairly constant. The presence of cytochrome P-448 in the mammary gland microsomes of 3-MC- or β-NF-treated lactating rats was also evidenced by binding spectra obtained with 2-FAA, a type I substrate for binding to the hepatic cytochrome P-448 [10]. The addition of 2-FAA to mammary gland microsomes of a β-NF-treated lactating rat gave a type I binding spectrum similar to that given by the hepatic microsomes, with the amplitude corresponding to the lower content of cytochrome P-448 in the mammary microsomes (Fig. 3).

The contents of cytochrome b_5 and cytochrome P-450 in the mammary gland microsomes of control (corn oil-treated) lactating rats were about 15 and 500 times less, respectively, than the contents of their hepatic microsomes (Table 2). Pretreatment of lactating rats with 3-MC or β-NF increased the content of cytochrome P-448 3- to 10-fold in the mammary gland microsomes and about 2-fold in the hepatic microsomes. The dose of the inducer, 20 or 40 mg of compound per kg of body weight, had only a marginal effect on the extent of the induction of cytochrome P-448 in the microsomes of both tissues and the stage of lactation was without effect on its induction. Pretreatment of rats with 3-MC or β-NF (40 mg/kg of body weight) increased 1.3-fold the content of cytochrome b_5 in the hepatic microsomes (Table 2). Whereas in the hepatic microsomes the values for total heme nearly equaled the sums of cytochrome b_5 and cytochrome P-450(P-448), in the mammary gland microsomes the values for total heme were much greater than the sums of these cytochromes. We considered the possibility that mammary gland microsomal fraction contained a substance which partially degraded its cytochrome P-450(P-448). We therefore examined the effects of mammary gland microsomes on the hepatic cytochrome P-450(P-448) after incubations of equal amounts of microsomal protein from both tissues for periods of 4–16 hr at 4°. Because incubations with mammary gland microsomes were without effect on the spectral measurements of hepatic cytochrome P-450(P-448) (data not shown), we assumed that a degradation of cytochrome P-450(P-448) in the mammary gland microsomes probably did not occur and that the relatively high values for total heme are from contamination of this fraction with cytochrome aa_3 and possibly catalase and residual hemoglobin. In addition, cytochrome P-420 has been reported in the mammary gland microsomes [7] and could contribute to the total heme values.

A comparison of the NADH-cytochrome *c* reduc-

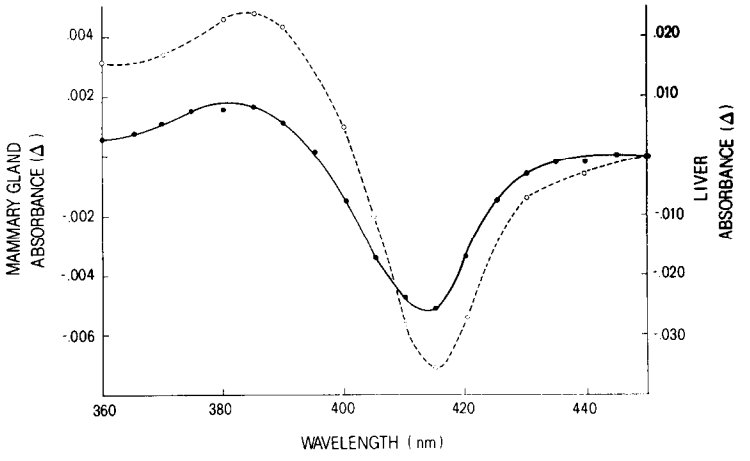

Fig. 3. Binding spectra given by 2-FAA on addition to mammary gland (●——●) and liver (○----○) microsomes of a β-NF-treated lactating rat. Mammary gland or liver microsomes were suspended in 300 mM sodium phosphate buffer, pH 7.5, containing 50% glycerol (8.0 or 4.0 mg protein/ml respectively) and divided between two cuvettes (1.2 ml each). The baseline was then recorded. 2-FAA in propylene glycol (20 μl) was added to the sample cuvette at a concentration of 0.12 mM and the vehicle alone was added to the reference cuvette. The spectra were recorded after 30 min at room temperature with a Hitachi 110 computerized double beam ratio recording spectrophotometer with automatic baseline correction (2.2 nm band width).

tase activities indicated that the mammary gland microsomes had about 50-fold lower activity than the liver microsomes (Table 2). Because this assay depends on cytochrome b_5 and the mammary gland had a 15-fold lower content of cytochrome b_5, the reductase activity was also measured with potassium ferricyanide, which can accept electrons directly from the reductase [27]. In this assay, we used twice as much NADH to ensure that its concentration was not rate-limiting. Under these conditions, in which

Table 2. Effects of pretreatment of lactating rats with 3-MC and β-NF on the microsomal enzymes of the mammary gland and liver

No. of rats	Day of lactation	Pretreatment of rats*	Cytochrome† b_5	Cytochrome† P-450 (P-448)	Total heme†	NADH–cytochrome *c* reductase‡	NADH–$K_3Fe(CN)_6$ reductase‡	NADPH–cytochrome *c* reductase‡
Mammary gland								
3	4–6	Corn oil	22.8 ± 6.3	1.3 ± 0.1	72.3 ± 19.4	43.1 ± 10.7	639 ± 42	12.0 ± 3.1
4	8–9	Corn oil	19.7 ± 9.6	1.3 ± 0.8	44.4 ± 25.1	27.6 ± 11.4	883 ± 381	13.1 ± 4.2
4	11–14	Corn oil	22.2 ± 7.1	3.0 ± 1.2	75.8 ± 16.9	26.6 ± 11.3	779 ± 212	18.8 ± 3.2
3	5–6	3-MC§	30.3 ± 3.2	12.0 ± 4.0‖	104 ± 32	37.9 ± 6.0	710 ± 141	19.6 ± 1.0
3	8–9	3-MC§	24.2 ± 2.3	7.8 ± 1.9	86.3 ± 33	29.9 ± 6.9	758 ± 129	20.4 ± 4.9
3	11	3-MC	19.4 ± 4.8	8.3 ± 3.3	97.0 ± 44	27.1 ± 6.9	872 ± 300	23.7 ± 5.4
3	7–10	β-NF§	18.7 ± 11.7	6.1 ± 4.3‖	115 ± 49	26.1 ± 6.5	587 ± 70	12.0 ± 5.4
7	8–9	β-NF	27.1 ± 10.2	13.9 ± 6.1	180 ± 77	31.6 ± 13.0	759 ± 233	18.1 ± 2.0
5	11–15	β-NF	17.9 ± 4.4	15.6 ± 7.7	129 ± 24	21.7 ± 3.7	881 ± 452	20.7 ± 3.7
Liver								
3	4–6	Corn oil	346 ± 15	554 ± 148	993 ± 75	1272 ± 176	3772 ± 399	173 ± 27
4	8–9	Corn oil	362 ± 74	533 ± 191	1181 ± 183	1310 ± 437	5350 ± 1336	160 ± 90
4	11–14	Corn oil	319 ± 75	548 ± 88	1101 ± 250	1413 ± 98	6089 ± 1242	186 ± 25
3	5–6	3-MC§	414 ± 43¶	873 ± 146‖	1625 ± 71	1401 ± 32	3006 ± 1083	151 ± 90
3	8–9	3-MC§	461 ± 124	1050 ± 132	1600 ± 259	1314 ± 258	5177 ± 666	180 ± 25
3	11	3-MC	430 ± 48	1068 ± 187	1683 ± 164	1363 ± 145	4394 ± 1416	193 ± 35
2	7–10	β-NF§	373 ± 88	919 ± 274‖	1310 ± 28	2147 ± 172	3793 ± 374	208 ± 57
7	8–9	β-NF	440 ± 76¶	1046 ± 161	1620 ± 184	1418 ± 652	4884 ± 1249	203 ± 82
5	11–15	β-NF	359 ± 74	961 ± 137	1484 ± 79	1517 ± 377	5656 ± 1622	181 ± 43

* Rats received i.p. injections of the vehicle (corn oil) or of the compound (40 mg/kg of body weight) for 3 consecutive days, unless stated otherwise.

† Units are pmoles/mg protein. Values are means ± S.D.

‡ Units are nmoles of substrate reduced · (mg protein)$^{-1}$ · min^{-1}. Values are means ± S.D.

§ The dose of i.p. injected compound in corn oil was 20 mg/kg of body weight for 3 consecutive days.

‖ Differences between the combined groups of control (corn oil-treated) rats and the combined groups of 3-MC- or β-NF-treated rats were statistically significant at $P < 0.001$.

¶ Differences between the combined groups of control (corn oil-treated) rats and the combined groups of 3-MC-treated rats or the group of 7 β-NF-treated rats were statistically significant at $P < 0.01$.

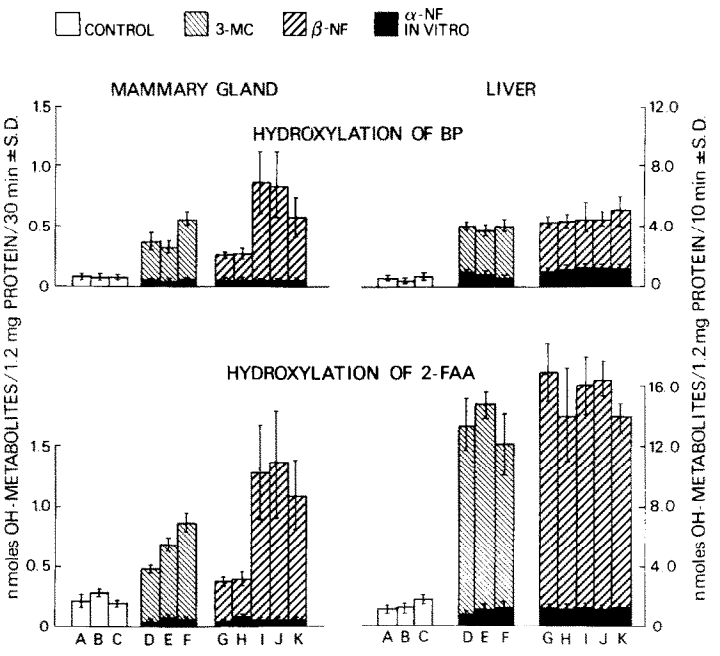

Fig. 4. Effects of pretreatment of lactating rats with 3-MC and β-NF on hydroxylation of BP and 2-FAA by mammary gland and liver microsomes. Rats received i.p. injections of the vehicle (corn oil) or of the compound (40 mg/kg of body weight) for 3 consecutive days before being killed unless stated otherwise. Groups A, B and C consisted of three, four and four rats each on days 4–6, 8–9 and 11–14 of lactation respectively. Groups D, E and F consisted of three rats each on days 5–6, 8–9 and 11 of lactation respectively. Rats in groups D and E received three i.p. injections of 3-MC at a dose level of 20 mg/kg of body weight. Groups G and H consisted of two rats each on days 7–10 and 3–4 of lactation respectively. Rats in groups G and H received three and two i.p. injections of β-NF at a dose level of 20 and 40 mg/kg of body weight respectively. Groups I, J and K consisted of seven, two and three rats each on days 8–9, 11 and 13–15 of lactation respectively. The differences in hydroxylation of both BP and 2-FAA between mammary gland and liver microsomes of control groups A, B and C were significant at $P < 0.001$. The differences in hydroxylation of both compounds by mammary gland and liver microsomes between groups A and D, B and E, and C and F were significant at $P < 0.001$. The differences in hydroxylation of both compounds by mammary gland and liver microsomes between groups A and H, B and G, B and I, C and J, and C and K were significant at $P < 0.001$, except for the differences in hydroxylation of 2-FAA by mammary gland microsomes between groups A and H, and B and G that were significant at $P < 0.025$. In the mammary gland microsomes, the differences in hydroxylation of BP between groups D and F ($P < 0.025$) and E and F ($P < 0.001$), and in hydroxylation of 2-FAA between groups D and F ($P < 0.001$) and E and F ($P < 0.005$), were significant. The differences in hydroxylation of both compounds between the groups G + H and I, G + H and J, and G + H and K were significant at $P < 0.005$. The inhibition of hydroxylation of BP and 2-FAA by 0.1 mM α-NF *in vitro* was significant in all groups examined at $P < 0.001$.

the reductase was rate-limiting, the mammary gland microsomes showed a 6-fold lower activity than the hepatic microsomes. These data indicated that both cytochrome b_5 and its NADH-dependent reductase activity were indeed much lower in the mammary gland microsomes. The NADPH-dependent cytochrome *c* reductase activity was also lower, about 10-fold, in the mammary gland than in the liver (Table 2). The measurements of reductase activities were rotenone-insensitive and were unaffected by the addition of cyanide. In neither mammary gland nor liver microsomes did the NADH- or NADPH-dependent reductase activities change after pretreatment of lactating rats with 3-MC or β-NF.

Hydroxylation of carcinogens may serve as a measure of mixed function oxidase activity. We measured hydroxylation of BP and 2-FAA in the mammary gland and liver microsomes of lactating rats on days 4–6, 8–9 and 11–14 of lactation (Fig. 4). The basal activity of the liver microsomes was 4- to 8-fold greater than that of mammary gland microsomes. The stage of lactation had no effect on the magnitude of hydroxylation of BP or 2-FAA. Pretreatment of lactating rats with 3-MC or β-NF increased hydroxylation of BP and 2-FAA about 15-fold in the hepatic microsomes. The magnitude of induction of the hydroxylating activity in the liver was independent of the dose of either inducer. By contrast, the induction of hydroxylation in mammary gland microsomes varied with the inducer and its dose. Pretreatment of rats with 3-MC, 20 and 40 mg compound per kg of body weight, increased hydroxylation of BP 6- and 10-fold, and of 2-FAA 2- and 4-fold, respectively. Pretreatment of rats with β-NF at the lower dose and the higher dose levels increased the hydroxylation of BP 4- and 16-fold, and of 2-FAA 2- and 6-fold, respectively. The optimal induction of hydroxylating activity in the mammary gland microsomes was measured between days 8 and 11 of lactation. The hydroxylation of BP and 2-FAA induced

by 3-MC or β-NF in the mammary gland and liver microsomes was inhibited by 0.1 mM α-NF *in vitro* (Fig. 4). This inhibitor had no significant effect on the basal hydroxylating activity of the mammary gland and liver microsomes (data not shown). Because α-NF is a specific inhibitor of cytochrome P-448-dependent aryl hydrocarbon hydroxylase in hepatic microsomes [28], these results indicated that the increased hydroxylating activities of mammary gland and liver microsomes of 3-MC- or β-NF-treated lactating rats were dependent on the cytochrome P-448 induced in these tissues.

DISCUSSION

Although mammary gland microsomes of lactating rats showed lower enzyme activities than their hepatic microsomes, a considerable redox potential was still indicated in this fraction. This confirmed the earlier reports of the presence of cytochrome b_5 and NADH- and NADPH-dependent reductase activities in the rat mammary gland [6, 7]. In the latter, cytochrome P-420 was also identified based on its characteristic absorbances in the carbon monoxide- and the dithionite-difference spectra and by the reaction with cyanide. We have observed similar spectral characteristics and ascribed them, at least in part, to a persistent contamination of mammary gland microsomes with bound hemoglobin. Since both hemoglobin and cytochrome P-420 were reduced by ascorbate and dithionite, but not NADH, and absorbed at 560–555 and 430–428 nm in the reduced state, and at 420–418 nm after complexation with carbon monoxide, it was difficult to distinguish between them. Moreover, cytochrome aa_3, which we found to be present in mammary gland microsomes, further complicated the interpretation of the spectra. Cytochrome aa_3 also interfered with our attempts to identify spectrally cytochrome P-420 complexes with cyanide.

Our earlier efforts to detect cytochrome P-450(P-448) in the mammary gland microsomes of the rat were unsuccessful chiefly because of instrument limitations to analyze spectrally microsomal suspensions in which protein concentration exceeded 2 mg per ml. Newer spectrophotometers with their improved optic systems have given us more accurate resolution and allowed us to analyze microsomal suspensions of 10 mg protein per ml. Therefore, our limit of detection of cytochrome P-450(P-448) improved from about 20 pmoles [8] to 0.5 pmole per mg of microsomal protein. On the other hand, the spectral interference by the contaminants, chiefly hemoglobin and cytochrome aa_3, in the concentrated microsomal suspensions increased and necessitated modifications in the preparation of microsomes as well as in the spectral assays. The procedure for eliminating interference by hemoglobin [29] and its application to the detection of cytochrome P-450 in rat mammary gland microsomes [30] were reported recently. With this method, we obtained a spectrum similar to that reported, with an absorbance maximum at 450–445 nm. However, further investigation indicated that this absorbance is not of the dithionite-reduced cytochrome P-450 complex with carbon monoxide, but of the dithionite-reduced cytochrome aa_3. Elimination of spectral interference by cytochrome aa_3 required reduction of mammary gland microsomes in both the reference and sample with sodium succinate in the presence of phenazine ethosulfate and sodium ascorbate as described here. We also found that the resolution of the absorbance of the reduced cytochrome P-450(P-448) complex with carbon monoxide in the mammary gland microsomes improved with time. This might be due to time-dependent complexation with carbon monoxide [25] and/or settling of microsomes after bubbling with gas.

In this work, we showed that the classical inducers of cytochrome P-448 in liver microsomes, 3-MC and β-NF [31], also induced cytochrome P-448 in the mammary gland microsomes of lactating rats. This is evidenced by the following data: (1) the absorbance at 448 nm of the carbon monoxide complex of the reduced mammary gland microsomes from 3-MC- or β-NF-treated lactating rats increased substantially; (2) 2-FAA, a type I binding substrate for the hepatic cytochrome P-448, produced a type I binding spectrum with mammary gland microsomes of 3-MC- or β-NF-treated rats; and (3) the hydroxylation of BP and 2-FAA increased markedly in the mammary gland and liver microsomes of lactating rats after their pretreatment with 3-MC or β-NF. The increased hydroxylating activities, but not the basal activities, were inhibited by α-NF *in vitro*, a specific inhibitor of cytochrome P-448-dependent aryl hydrocarbon hydroxylase [28]. The metabolic assays indicated that mammary gland microsomes of 3-MC- and especially those of β-NF-treated lactating rats had a substantial capacity for hydroxylation of the carcinogens. Since in this work we determined only the overall conversion of BP and 2-FAA to the hydroxy metabolites, and not their individual metabolite profiles, it is as yet unknown if any of the hydroxy products formed are carcinogenic. It is of particular interest to answer this question in the case of 2-FAA, the conversion of which to *N*-hydroxy-2-FAA determines its carcinogenicity for the rat mammary gland [8]. Mammary gland microsomes of lactating rats, chronically treated with 2-FAA, had a low capacity for *N*-hydroxylation of 2-FAA [32].

Mammary gland mitochondrial fraction had a cytochrome *c* oxidase activity similar to that of liver mitochondria. This enzyme has been shown to oxidize certain metabolites of 2-FAA, i.e. *o*-aminophenols, to *o*-quinoneimines that were capable of covalent binding to proteins *in vitro* [33]. Because such interactions are implicated in carcinogenesis, this oxidative metabolic pathway leading to a reactive species remains to be investigated in mammary gland.

Acknowledgements—We thank Mr. Richard Decker and Mr. Donald Olson for their valuable technical assistance, and Mr. Scott Nelson for the electron microscopy. We also thank Dr. Daune L. Crankshaw for helpful discussions. The use of a Varian Cary 219 spectrophotometer at the Royal A. and Olive W. Stone Research Laboratories, University of Minnesota, is gratefully acknowledged.

REFERENCES

1. E. I. Ciaccio and H. DeVera, *Biochem. Pharmac.* **25**, 985 (1976).
2. J. M. Fysh and A. B. Okey, *Biochem. Pharmac.* **27**, 2972 (1978).
3. J. M. Fysh and A. B. Okey, *Can. J. Physiol. Pharmac.* **57**, 112 (1979).
4. K. M. McCormack, P. Melrose, D. E. Rickert, J. G. Dent, J. E. Gibson and J. B. Hook, *Toxic. appl. Pharmac.* **47**, 95 (1979).
5. J. W. Greiner, A. H. Bryan, L. B. Malan-Shibley and D. H. Janss, *J. natn. Cancer Inst.* **64**, 1127 (1980).
6. E-D. Jarasch, G. Bruder, T. W. Keenan and W. W. Franke, *J. Cell Biol.* **73**, 223 (1977).
7. G. Bruder, A. Fink and E.-D. Jarasch, *Expl Cell Res.* **117**, 207 (1978).
8. D. Malejka-Giganti, R. E. Rydell and H. R. Gutmann, *Cancer Res.* **37**, 111 (1977).
9. S. Orrenius, Å. Ellin, S. V. Jakobsson, H. Thor, D. L. Cinti, J. B. Schenkman and R. W. Estabrook, *Drug Metab. Dispos.* **1**, 350 (1973).
10. D. Malejka-Giganti, R. C. McIver, A. L. Glasebrook and H. R. Gutmann, *Biochem. Pharmac.* **27**, 61 (1978).
11. T. Matsubara, R. A. Prough, M. D. Burke and R. W. Estabrook, *Cancer Res.* **34**, 2196 (1974).
12. D. Malejka-Giganti, A. H. Potter and R. E. Rydell, *Lab. Invest.* **42**, 627 (1980).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
15. W. H. Vanneste, *Biochim. biophys. Acta* **113**, 175 (1966).
16. T. Matsubara, M. Koike, A. Touchi, Y. Tochino and K. Sugeno, *Analyt. Biochem.* **75**, 596 (1976).
17. K. G. Paul, H. Theorell and A. Akeson, *Acta chem. scand.* **7**, 1284 (1953).
18. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
19. E. G. Hrycay and R. A. Prough, *Archs Biochem. Biophys.* **165**, 331 (1974).
20. T. K. Hodges and R. T. Leonard, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. XXXII, part B, p. 392. Academic Press, New York (1974).
21. J. E. Brown and D. Kupfer, *Chem. Biol. Interact.* **10**, 57 (1975).
22. E. J. Barry, D. Malejka-Giganti and H. R. Gutmann, *Chem. Biol. Interact.* **1**, 139 (1969/70).
23. J. H. Wang, in *Oxygenases* (Ed. O. Hayaishi), p. 469. Academic Press, New York (1962).
24. W. W. Wainio, *J. biol. Chem.* **212**, 723 (1955).
25. J. G. Ghazarian, C. R. Jefcoate, J. C. Knutson, W. H. Orme-Johnson and H. F. DeLuca, *J. biol. Chem.* **249**, 3026 (1974).
26. W. Cammer and R. W. Estabrook, *Archs Biochem. Biophys.* **122**, 735 (1967).
27. P. Strittmatter, H. G. Enoch and P. Fleming, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. LII, Part C, p. 206. Academic Press, New York (1978).
28. F. J. Wiebel and H. V. Gelboin, *Biochem. Pharmac.* **24**, 1511 (1975).
29. K. A. M. Johannesen and J. W. DePierre, *Analyt. Biochem.* **86**, 725 (1978).
30. L. E. Rikans, D. D. Gibson and P. B. McCay, *Biochem. Pharmac.* **28**, 3039 (1979).
31. A. R. Boobis, D. W. Nebert and J. S. Felton, *Molec. Pharmac.* **13**, 259 (1977).
32. D. Malejka-Giganti, *J. natn. Cancer inst.* Mono No. 70, in press.
33. H. R. Gutmann and H. T. Nagasawa, *J. biol. Chem.* **235**, 3466 (1960).