gans that secrete proteins (pituitary, pancreas, thyroid, thymus, liver, and salivary gland) was incubated under conditions identical to the parathyroid incubation, less than 0.5 percent of the radioactive protein in the medium had the electrophoretic mobility of PSP. If PSP is unique to the parathyroid gland, it may be the basis of a new assay for parathyroid gland activity. Since PSP is a large protein, it is likely to be highly immunogenic. A radioimmunoassay for PSP might be developed to complement that for PTH, a situation analogous to the sensitive radioimmunoassay for neurophysin which measures the secretory activity of the posterior pituitary gland (*19*).

BYRON KEMPER
Department of Biology, Massachusetts Institute of Technology, Cambridge 02139
JOEL F. HABENER
Endocrine Unit and *Department of Medicine, Massachusetts General Hospital,* and *Harvard Medical School, Boston 02114*
ALEXANDER RICH
Department of Biology, Massachusetts Institute of Technology
JOHN T. POTTS, JR.
Endocrine Unit and *Department of Medicine, Massachusetts General Hospital,* and *Harvard Medical School*

References and Notes

1. D. W. Fawcett, J. A. Long, A. L. Jones, *Recent Prog. Horm. Res.* **25**, 315 (1969); L. J. Greene, C. H. W. Hirs, G. E. Palade, *J. Biol. Chem.* **238**, 2054 (1963).
2. W. W. Douglas, *Pharmacol. Rev.* **18**, 471 (1966); O. H. Viveros, L. Arqueros, N. Kishner, *Life Sci.* **7**, 609 (1968); H. Blaschko, R. S. Comline, F. W. Schneider, M. Silver, A. D. Smith, *Nature (Lond.)* **215**, 58 (1967); P. Banks and K. Helle, *Biochem. J.* **97**, 40c (1965).
3. A. H. Rubenstein, J. L. Clark, F. Melani, D. F. Steiner, *Nature (Lond.)* **224**, 697 (1969).
4. C. P. Fawcett, A. E. Powell, H. Sachs, *Endocrinology* **83**, 1299 (1968).
5. B. Kemper, J. F. Habener, J. T. Potts, Jr., A. Rich, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 643 (1972).
6. L. Ornstein and B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964).
7. U. K. Laemmli, *Nature (Lond.)* **227**, 680 (1970).
8. L. G. Raisz, *ibid.* **197**, 1115 (1963); A. D. Care, L. M. Sherwood, J. T. Potts, Jr., G. D. Aurbach, *ibid.* **209**, 55 (1966).
9. W. Y. W. Au, A. P. Poland, P. H. Stern, L. G. Raisz, *J. Clin. Invest.* **49**, 1639 (1970); L. M. Sherwood, J. S. Rodman, W. B. Lundberg, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1631 (1970); L. M. Sherwood, M. Abe, J. S. Rodman, W. B. Lundberg, Jr., J. H. Targovnik, in *Calcium, Parathyroid Hormone and the Calcitonins,* proceedings of the Fourth Parathyroid Conference, R. V. Talmage and P. L. Munson, Eds. (Excerpta Medica, Amsterdam, 1972), pp. 183–196.
10. A. A. Licata, W. Y. W. Au, L. G. Raisz, *Biochim. Biophys. Acta* **261**, 143 (1972).
11. R. S. Yalow and S. A. Berson, *Biochem. Biophys. Res. Commun.* **48**, 391 (1972).
12. ———, *ibid.* **44**, 439 (1971).
13. ———, *Metabolism* **22**, 703 (1973).
14. M. E. Stachura and L. A. Frohman, *Endocrinology* **92**, 1708 (1973); A. D. Goodman, R. Tanenbaum, D. Rabinowitz, *J. Clin. Endocrinol. Metab.* **35**, 868 (1972).
15. S. B. Oldham, J. A. Fisher, C. D. Arnaud, *J. Lab. Clin. Med.* **78**, 821 (1971).
16. V. Herbert, K. D. Lau, C. W. Gottlieb, S. J. Bleicher, *J. Clin. Endocrinol. Metab.* **25**, 1375 (1965). Charcoal is coated with dextran, which selectively allows small peptides and not large proteins to penetrate and adsorb to the charcoal particle.
17. D. V. Cohn, R. R. MacGregor, L. L. H. Chu, J. W. Hamilton, in *Calcium, Parathyroid Hormone and Calcitonins,* proceedings of the Fourth Parathyroid Conference, R. V. Talmage and P. L. Munson, Eds. (Excerpta Medica, Amsterdam, 1972), pp. 173–182; D. V. Cohn, R. R. MacGregor L. L. H. Chu, J. R. Kimmel, J. W. Hamilton, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1521 (1972).
18. J. W. Hamilton, H. D. Niall, H. T. Keutmann, J. Jacobs, J. T. Potts, Jr., D. V. Cohn, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
19. K. W. Cheng and H. G. Friesen, *Endocrinology* **88**, 608 (1971); M. J. Martin, T. Chard, J. Landon, *J. Endocrinol.* **52**, 481 (1972).
20. J. W. Hamilton, F. W. Spierto, R. R. MacGregor, D. V. Cohn, *J. Biol. Chem.* **246**, 3224 (1971). Losses of PTH occur during column chromatography at neutral *p*H, but not in the 8*M* urea solutions containing acetic acid or hydrochloric acid which are used in polyacrylamide gel electrophoresis.
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Benzo[a]pyrene Metabolites: Efficient and Rapid Separation by High-Pressure Liquid Chromatography

Abstract. *High-pressure liquid chromatography can separate eight metabolites of benzo*[a]*pyrene formed by rat liver microsomes. This method offers major advantages over previous techniques used for the separation of oxygenated polycyclic aromatic hydrocarbons.*

Polycyclic aromatic hydrocarbons (PAH's), which cause cancer in experimental animals, are found in the atmosphere, waterways and oceans, soil, marine life, and in the food chain. Sources of these pollutants include emissions from transportation, heat and power generation, refuse burning, industrial processes, and oil spills (*1*). Mammals and many lower organisms metabolize polycyclic hydrocarbons, primarily by enzymatic oxygenation, a process which converts the hydrocarbons into organic solvent–soluble polycyclic phenols, dihydrodiols, quinones, and water-soluble conjugates (*2, 3*). Some of these metabolites are formed through epoxide intermediates (*2*). These PAH's are also covalently bound to cellular macromolecules (*4*). Oxygenation is primarily catalyzed by a microsomal enzyme complex aryl hydrocarbon hydroxylase which contains cytochrome P-450. This enzyme complex has a major function in both the detoxification of PAH's (*5*) and the activation of some to toxic derivatives or to active carcinogens (*6*). In order to understand the relation between the formation of specific metabolites and the induction of cancer, it is necessary to describe for each PAH its profile of metabolites and to characterize the biological activity of each product. Thin-layer chromatography or column chromatography have been the usual methods of separation (*3, 7*). Although useful in specific cases, these methods are often inadequate. The separations are often incomplete; because the metabolites are often labile when exposed to light and air, they are easily destroyed. Gas chromatography has been used successfully for the separation of the parent PAH, but is inadequate for the hydroxylated metabolites, which are destroyed by pyrolysis during the vaporization stage. Recently, trimethylsilylation of methylcholanthrene metabolites has permitted their

Table 1. High-pressure liquid chromatographic separation of benzo[*a*]pyrene metabolites. The following are abbreviations used: BP, Benzo[*a*]pyrene; 9,10-diol, 9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene; 7,8-diol, 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene; 4,5-diol, 4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene; 3-OH, 3-hydroxybenzo[*a*]pyrene; 9-OH, 9-hydroxybenzo[*a*]pyrene; 1,6-quinone, Benzo[*a*]pyrene-1,6-dione; 3,6-quinone, benzo[*a*]pyrene-3,6-dione; 6,12-quinone, benzo[*a*]pyrene-6,12-dione.

Metabolite	Retention time (min)	MW*
9,10-diol-BP	8.5	286
4,5-diol-BP	15.5	286
7,8-diol-BP	18.0	286
1,6-quinone-BP	25.5	282
3,6-quinone-BP	26.0	282
6,12-quinone-BP†	28.0	
9-OH	35.0	268
3-OH	37.0	268
BP	48.0	252

* Molecular weight (MW) determinations were performed on a Jeol JMS-01SG-2 at 70 electron volts, with a solid probe; the temperature ranged from 90° to 150°C. † Insufficient material for complete analysis, and therefore tentative.

separation by gas chromatography (*8*).

We now report that high-pressure liquid chromatography is an efficient, rapid, and reproducible method for the separation of metabolites of benzo[*a*]pyrene. Benzo[*a*]pyrene is found throughout the environment and is used as the prototype for the presence of PAH (*1*). Our results with this technique indicate that it will be generally useful for the separation of metabolites of other PAH's.

Metabolites of benzo[*a*]pyrene were formed by incubation of [^{3}H]- or [^{14}C]-benzo[*a*]pyrene with liver microsomes prepared from rats that had been treated with methylcholanthrene. The organic solvent–soluble metabolites produced by incubation were extracted, concentrated by evaporation, and injected into the liquid chromatograph. The various peaks obtained by liquid chromatography were isolated and identified by their mass spectra and ultraviolet absorption spectra, and by comparison with metabolites isolated and characterized by thin-layer chromatography (TLC) (*7*).

The distribution of the ultraviolet absorption at 254 nm and radioactivity in Fig. 1A shows eight distinct bands in addition to the parent benzo[*a*]pyrene. None of these bands except the benzo[*a*]pyrene band appeared in the analysis of unincubated preparations. Figure 1, B to G, shows the distribution of [^{14}C]benzo[*a*]pyrene metabolites that were isolated by TLC and injected with the mixture of [^{3}H]benzo[*a*]pyrene metabolites. The ultraviolet absorption and ^{3}H-labeling profile in Fig. 1, B to G, were identical to those of Fig. 1A and they have been omitted in order to facilitate the reading of the ^{14}C-labeled peaks. As is shown in Fig. 1A, the 9,10-diol, 4,5-diol, and 7,8-diol are completely separated by liquid chromatography, whereas they are often incompletely resolved when TLC is used. The 3-OH and 9-OH metabolites are not separable by TLC; however, the single spot obtained from TLC is separated into two distinct peaks by liquid chromatography (Fig. 1C). Thus, the two phenols are easily distinguishable. The 1,6- and the 3,6-quinones are completely separated from the phenols and diols, but not entirely separated from each other (Fig. 1D). Figure 1A also indicates a third quinone not previously seen. We have tentatively identified it as the 6,12-quinone by co-chromatography with an authentic sample. Table 1 summarizes the retention times and mass spectral data for each of the peaks and, together with Fig. 1, indicates the basis for their identification.

The method reported above offers an important advance in this field and should substantially increase the progress in this area of research.

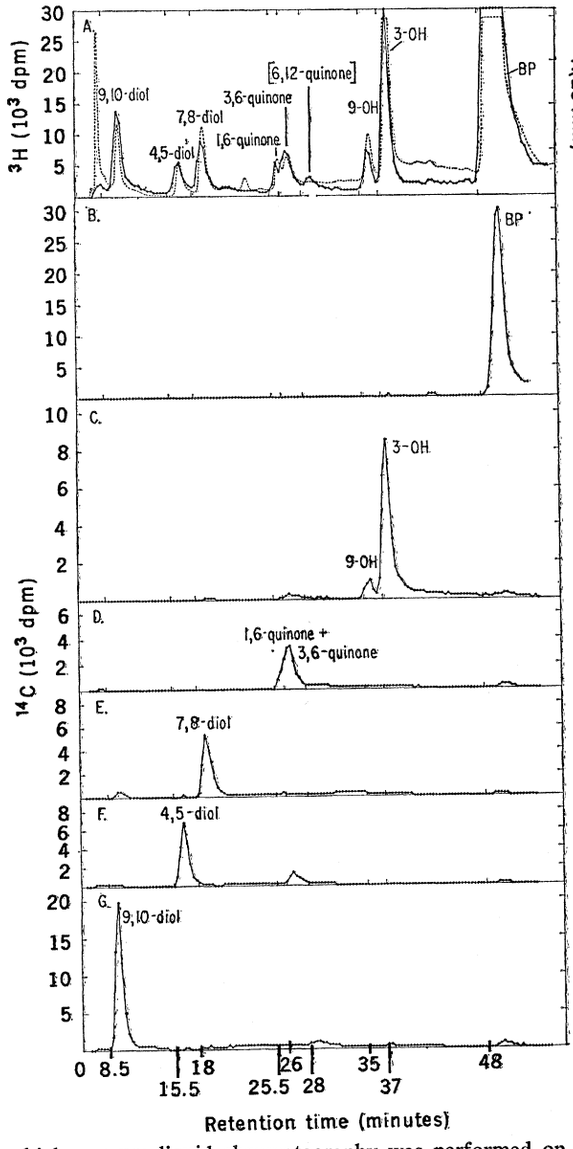

Fig. 1. Separation of metabolites of benzo[*a*]pyrene. Male Sprague-Dawley rats (160 to 180 g) were injected intraperitoneally with 5 mg of 3-methylcholanthrene in 0.5 ml of corn oil; 40 hours later the rats were killed. Liver microsomes were prepared as described (*7*). The metabolites were formed by incubating rat liver microsomes with [^{3}H]-benzo[*a*]pyrene (specific activity, 70 mc/mmole) or [^{14}C]benzo[*a*]pyrene (21 mc/mmole; Amersham/Searle) as follows. Each flask contained in a total volume of 1.0 ml: 1 to 2 mg of microsomal protein, 0.36 μmole of reduced nicotinamide adenine dinucleotide phosphate, 3 μmole of $MgCl_2$, 50 μmole of tris-HCl buffer, *p*H 7.5, and 100 nmole of benzo[*a*]pyrene dissolved in 0.040 ml of methanol. The flasks were incubated for 30 minutes at 37°C under red light illumination, and the reaction was stopped by the addition of 1.0 ml of acetone. The mixture was then extracted with 2.0 ml of ethyl acetate; five extracts were pooled and dried over 1.0 g of anhydrous magnesium sulfate, the solvent was evaporated under vacuum, and the residue (metabolites) was dissolved in 0.1 ml of methanol. Isolation and characterization of the ^{14}C-labeled metabolites by TLC has been described (*7*). The high-pressure liquid chromatography was performed on a high-pressure liquid chromatograph (Dupont model 830) fitted with a permaphase column (1-m ODS). The column was eluted with a reverse phase gradient system, with methanol and water (30 : 70 initially, and 70 : 30 at the end). The gradient rate of change was 3 percent per minute; the column temperature was 50°C; the pressure was 350 pounds per square inch; flow rate was 0.6 ml/min. The eluate was monitored by ultraviolet absorption at 254 nm. Fractions were collected at 20-second intervals, and the radioactivity was determined in a Beckman 350 scintillation counter with Aquasol (New England Nuclear) as the counting medium. (A) —, ^{3}H-labeling, measured by 10^3 disintegrations per minute; - - - -, ultraviolet absorption. (B to G), ^{14}C.

JAMES K. SELKIRK, ROBERT G. CROY
HARRY V. GELBOIN

Molecular Carcinogenesis Section, Chemistry Branch, National Cancer Institute, Bethesda, Maryland 20014

References and Notes

1. Anonymous, *Particulate Polycyclic Organic Matter* (National Academy of Sciences, Washington, D.C., 1972).
2. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltman-Nirenberg, S. J. Udenfriend, *Biochemistry* **9**, 147 (1970); J. K. Selkirk, E. Huberman, C. Heidelberger, *Biochem. Biophys. Res. Commun.* **43**, 1010 (1971); P. L. Grover, A. Hewer, P. Sims, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **18**, 76 (1971).
3. P. Sims, *Biochem. Pharmacol.* **16**, 613 (1967); **19**, 795 (1970); A. H. Conney, E. C. Miller, J. Miller, *J. Biol. Chem.* **228**, 753 (1957); H. L. Falk, P. Kotin, S. S. Lee, A. Nathan, *J. Natl. Cancer Inst.* **28**, 699 (1962); L. Diamond, C. Sardet, G. M. Rothblat, *Int. J. Cancer* **3**, 838 (1968); E. Huberman, J. K. Selkirk, C. Heidelberger, *Cancer Res.* **31**, 2161 (1971).
4. P. Brooks and P. D. Lawley, *Nature (Lond.)* **202**, 781 (1964); C. Heidelberger and M. G. Moldenhauer, *Cancer Res.* **16**, 442 (1956); E. C. Miller, *ibid.* **11**, 100 (1951); S. A. Lesko, H. D. Hoffman, P. O. P. Ts'o, V. M. Maher, *Progress in Molecular and Subcellular Biology*, F. E. Hahn, Ed. (Springer-Verlag, New York, 1971),

vol. 2, pp. 347–350; T. Kuroki, E. Huberman, H. Marquardt, J. K. Selkirk, C. Heidelberger, P. L. Grover, P. Sims, *Chem.-Biol. Interactions* **4**, 389 (1971/19[illegible] → A. Borgen, H. Darvey, N. Castagnoli, T. T. Crocker, R. E. Rasmussen, I. Y. Wang, *J. Med. Chem.* **16**, 502 (1973); H. V. Gelboin, *Cancer Res.* **29**, 1272 (1969).
5. L. W. Wattenberg, *Cancer Res.* **26**, 1520 (1966); H. V. Gelboin, *Adv. Cancer Res.* **10**, 1 (1967).
6. H. V. Gelboin, E. Huberman, L. Sachs, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1188 (1969); H. V. Gelboin, F. J. Wiebel, L. Diamond, *Science* **170**, 169 (1970); H. V. Gelboin, N. Kinoshita, F. J. Wiebel, *Fed. Proc.* **31**, 1298 (1972).
7. N. Kinoshita, B. Shears, H. V. Gelboin, *Cancer Res.* **33**, 1937 (1973).
8. T. A. Stoming and E. Bresnick, *Science* **181**, 951 (1973).
9. We thank Dr. Peter Roller for performing the mass spectral analyses of the metabolites.

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Human Chromosome Banding by Feulgen Stain Aids in Localizing Classes of Chromatin

Abstract. *The band patterns of human chromosomes displayed by the Feulgen stain are similar to but not identical with those of the conventional quinacrine and Giemsa preparations. The parallelism among the three is principally that of prominent negative bands that appear consistently at characteristic loci throughout a wide range of chromosome compaction. The correlation of those bands with regions of low optical density in nonbanded Fuelgen-stained chromosomes suggests that they are loci of DNA that is inherently diffuse or readily labile. The instances of disparity among the patterns of the three modes of banding occur at the telomeres and at known regions of heterochromatin, and are interpreted here as reflecting the heterogeneity of the protein moieties in their reactivity to the cytochemical treatments.*

As has been demonstrated for the chromosomes of the mouse (*1*), the correspondence among the Feulgen banding patterns of human chromosomes (F bands) and those produced by other methods in general use—quinacrine fluorescence (Q bands) and Giemsa chromaticity (G bands)—is sufficiently close for pairing and identification of F banded chromosomes to be made in terms of the standard karyotypes and idiogram established for the Q and G bands (*2*). On analysis, the correspondence proves to be based principally on certain negative regions that are constant and so characteristic that they have been designated as "landmarks" (*2*). However, there are some chromosome segments in the complements of both mouse and man where the tripartite correspondence does not persist. Those regions in the chromosomes of man are well defined in the Feulgen banded karotype and have been identified as heterochromatin (*3*) or highly repetitious DNA, a fraction that has been found by biochemical analysis to display considerable heterogeneity with respect to base ratios (*3, 4*). With the parameter of DNA density established by the specificity and stoichiometry of the Feulgen reaction, a study of the instances of correspondence and disparity in the banding patterns produced by the three cytological stains allows for localization of different classes of chromatin and provides a basis for their cytochemical characterization. My observations provide support for the hypothesis (*1*) that the mechanism of banding, analyzed in terms of the Feulgen method, is bimodal. The negative bands are those inherently sparse in DNA or those from which the DNA has been removed by one or several steps of the cytochemical procedure, while the dark bands result from precipitation in situ of a selective group of chromatinic proteins with the consequent condensation of their associated DNA.

Lymphocytes were harvested after 3 to 4 days of culture with and without a 2-hour incubation with Colcemid. Chromosome spreads were prepared as described (*1*). Some of each set of slides were treated prior to Feulgen staining to induce banding (*1*). Karyotypes were assembled from micrographs of well-spread metaphase sets of varying degrees of compaction. Designation of chromosome number was made by reference to the standard karyotype (*2*) and to those appearing in original publications (*5–9*). Of the preparations obtained from seven series, representing four subjects, 40 complete metaphase sets that had been treated for banding were analyzed for the presence of the characteristic negative bands. Those included preparations that had been treated with Colcemid and preparations not treated with Colcemid, with values for total length of the chromosome complement ranging from 163 to 335 units (*10*).

The parallelism between the prominent negative regions of the Feulgen banded preparations and those of the standard Q and G banded chromosomes is reported in Table 1. With one exception, 2q distal, each of the negative regions designated as "landmarks" (*2*) of the Q and G bands (Table 1, column A) was detectable in one or both homologs of each chromosome pair of all Feulgen banded sets examined

Table 1. Characteristic negative bands of human chromosomes. (Column A) Characteristic and consistent negative bands of both G and Q patterns of human chromosomes. No marking (no asterisk) indicates designated negative landmarks (*2*). The (*) indicates other regions consistently seen as negative (*21*). (Column B) Negative regions detected in one or both homologs in 40 Feulgen banded human karotypes (Fig. 1). (Column C) Negative or pale regions seen in a nonbanded Feulgen stained set (Fig. 2, series b).

Arm	A	B	C
1p	Distal (tip)*	Distal	Distal
q	Proximal	Proximal	Proximal
2p	Median	Median	Median-distal
q	Proximal	Proximal	
	Distal		
3p	Median	Median	Median
q	Median	Median-distal	Median-distal
4q	Proximal	Proximal	Proximal
	Distal	Distal	
5q	Distal	Distal	
6p	Median	Median	Median
q	Median	Median	
7q	Median*	Median	Median
8p	Median	Median	Median
9q	Median*	Median	
10q	Proximal*	Proximal	Proximal
11q	Median	Median	Median
12q	Proximal*	Proximal	Proximal
14q	Median-distal*	Median-distal	Median
15q	Median*	Median	Median
17q	Proximal*	Proximal	Proximal
18q	Median	Median	Median