cubation in a nitrogen atmosphere, or in the presence of carbon monoxide, also resulted in little or no enzymatic activity. These results indicate that the skin enzyme requires oxygen and NADPH for its catalytic activity, as has been found for the liver microsomal enzyme system which hydroxylates BP (*1*).

Variation in the inducibility of BP hydroxylase obtained in skin samples from different newborn infants may, among other reasons, be due to genetic factors in the neonates, as well as to differences in exposure of the mother during gestation to drugs, environmental hydrocarbons, and so forth. Further studies will be necessary to evaluate the relative contributions of these factors to the inducibility of BP hydroxylase in human skin, and to determine whether the control amount and inducibility of this enzyme in skin will be predictive of the ability of humans to metabolize BP and other environmental carcinogens.

W. LEVIN
A. H. CONNEY
Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, New Jersey 07110
A. P. ALVARES
I. MERKATZ, A. KAPPAS
Rockefeller University and
Cornell University Medical College, New York 10021

References and Notes

1. A. H. Conney, E. C. Miller, J. A. Miller, *J. Biol. Chem.* **228**, 753 (1957).
2. L. W. Wattenberg and J. L. Leong, *J. Histochem. Cytochem.* **10**, 412 (1962); A. H. Conney, *Pharmacol. Rev.* **19**, 317 (1967).
3. R. M. Welch, Y. E. Harrison, A. H. Conney, P. J. Poppers, M. Finster, *Science* **160**, 541 (1968); R. M. Welch, Y. E. Harrison, B. W. Gommi, P. J. Poppers, M. Finster, A. H. Conney, *Clin. Pharmacol. Ther.* **10**, 100 (1969); D. W. Nebert, J. Winkler, H. V. Gelboin, *Cancer Res.* **29**, 1763 (1969); M. R. Juchau, *Toxicol. Appl. Pharmacol.* **18**, 565 (1971); A. H. Conney, R. M. Welch, R. Kuntzman, A. Poland, P. J. Poppers, M. Finster, J. A. Wolff, A. D. Munro-Faure, A. W. Peck, A. Bye, R. Chang, M. Jacobson, *Ann. N.Y. Acad. Sci.* **179**, 155 (1971).
4. J. H. Weisburger, P. H. Grantham, E. Vanhorn, N. H. Steigbigel, D. P. Rall, E. K. Weisburger, *Cancer Res.* **24**, 475 (1964).
5. B. B. Brodie, in *Drug Responses in Man*, G. E. W. Wolstenholme and R. Porter, Eds. (Little, Brown, Boston, 1967), p. 188; E. S. Vesell, G. T. Passananti, F. E. Greene, J. G. Page, *Ann. N.Y. Acad. Sci.* **179**, 752 (1971).
6. G. N. Wogan, *Fed. Proc.* **27**, 932 (1968); N. D. McGlashan, C. L. Walters, A. E. M. McLean, *Lancet* **1968-II**, 1017 (1968); L. S. DuPlessis, J. R. Nunn, W. A. Roach, *Nature* **222**, 1198 (1969); F. Homburger, E. Boger, *Cancer Res.* **28**, 2372 (1968); H. L. Falk, P. Kotin, *Clin. Pharmacol. Ther.* **4**, 88 (1963); E. L. Wynder and D. Hoffman, *Tobacco and Tobacco Smoke* (Academic Press, New York, 1967), pp. 336–339.
7. D. W. Nebert and H. V. Gelboin, *J. Biol. Chem.* **243**, 6242 (1968).
8. L. J. Alfred and H. V. Gelboin, *Science* **157**, 75 (1967); D. W. Nebert and H. V. Gelboin, *J. Biol. Chem.* **243**, 6250 (1968); *ibid.* **245**, 160 (1970); J. J. Giden and D. W. Nebert, *Science* **172**, 167 (1971).
9. Supported in part by PHS grants HD 04313 and ES 0621 and a grant from the Council for Tobacco Research-USA. We thank Susan Leigh for technical assistance. We thank Dr. H. V. Gelboin for the authentic standard of 3-hydroxybenzo[α]pyrene. We thank Mrs. MaryAnn Sadvary for her help in the preparation of this manuscript.

6 December 1971; revised 27 January 1972 ■

Interactions of Immunoglobulins G and M in the Detection of the Mammalian C-Type Virus Cross-Reactive Antigen

Abstract. *The mammalian C-type tumor viruses share an antigenic determinant, gs-3, located on the major internal polypeptide of the virion. Detection of this determined in gel diffusion assays by antiserums prepared in rats by immunization with rat tumor homogenates carrying murine virus and serums prepared in a rabbit by immunization with purified murine gs antigen depended on antibodies present in the fractions containing immunoglobulins M and G. The immunoglobulin G fraction by itself precipitated only the homologous murine antigen. Neither fraction alone precipitated heterologous (cat, rat, or hamster) antigen (definition of the gs-3 reaction), while a mixture of the two fractions did. The gs-3 reaction was eliminated by treatment of the serums with β-mercaptoethanol, also indicating a requirement for immunoglobulin M antibodies.*

The major internal protein (group-specific, gs, protein) of four mammalian C-type viruses carries both species-specific (gs-1) (*1*) and cross-reactive, interspecific (gs-3) (*2*) antigenic determinants (*3–5*). The strongest evidence for this conclusion derives from precipitation experiments with internally labeled, highly purified feline leukemia virus (FeLV) gs protein. In these experiments, both guinea pig antiserum to feline virus specific gs-1 and rat serum prepared against tumors carrying murine sarcoma virus and containing gs-3 antibody, precipitated 100 percent of the labeled antigen preparation (*6*). Nonetheless, in the course of analysis of antiserums prepared against purified gs protein or of antiserums from animals immunized with tumor homogenates containing murine sarcoma virus, occasional double preciptin lines were observed in gel diffusion assays even with highly purified antigen preparations. Among the possibilities considered to explain this observation was the variable occurrence of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies in the various serums. Our data support this hypothesis and further show an unexpected synergism of IgM and IgG fractions for visualization of the gs-3 reaction in agar gel diffusion.

The various C-type viruses were purified from chronically infected tissue cultures, and the gs proteins were obtained by isoelectric focusing as described (*4, 5, 7, 8*).

The antiserums used in the present study were rabbit antiserum to the purified gs protein from murine C-type virus (MuLV) prepared by multiple inoculations of purified protein, and rat antiserum prepared by multiple injections of Fisher rats with homogenates of murine sarcoma virus–induced tumors transplanted in the Fisher strain (*3*).

The two antiserums and normal rat and rabbit serums were separated into IgM- and IgG-containing fractions by gel filtration with Sephadex G-200 (*9*). The buffer system used was 0.02*M* tris-HCl, *p*H 8.2, containing 0.2*M* NaCl. Reduction and alkylation of IgM fractions were carried out as follows. One volume of 1.0*M* β-mercaptoethanol was added to nine volumes of serum and the mixture was incubated for 2 hours at room temperature. Serums were then alkylated by dialysis against 0.02*M* iodoacetamide for 16 hours in the cold (*10*).

Gel diffusion was made on microscope slides with 0.8 percent agarose in 0.05*M* tris buffer, *p*H 7.4, containing 0.1*M* NaCl and merthiolate (1 : 10,000) as a preservative. Complement fixation (CF) was carried out by the microtiter procedure as used in this laboratory (*11*).

The ability of the rat antiserums (designated MSV-I) to detect gs-3 in gel diffusion assays has been reported (*3*). The immunization procedure results in essentially a 100 percent response to gs-3 determinants, whereas only a rare tumor-bearing animal gives adequate gs-3 antiserums (*2*). The rabbit antiserum against MuLV gs protein also detected the gs-3 determinant, as well as the MuLV gs-1 determinant, and in this respect differs at least quantitatively from guinea pig antiserums which were mainly reactive with gs-1 determinants (*3, 4, 5, 8*). However, as

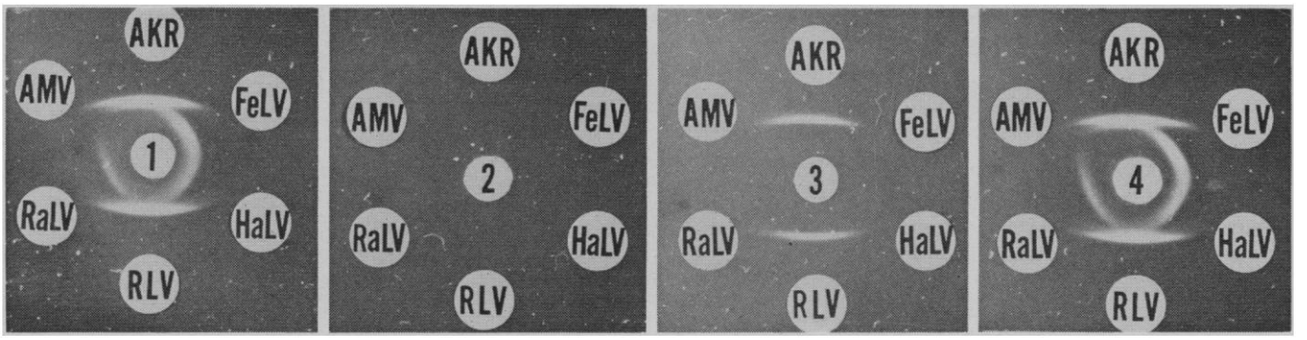

Fig. 1. Precipitin reactions of C-type virus gs antigens with rat antiserum MSV-I) and its fractions. Peripheral wells: Tween-ether disrupted C-type viruses as indicated (*AMV*, avian myeloblastosis virus; obtained from Dr. P. Sarma). Center wells contain (1) unfractionated MSV-I serum, (2) serum fraction that contains IgM (19*S* antibodies), (3) serum fraction that contains IgG (7*S* antibodies), and (4) a mixture (1 : 1) of IgM and IgG fractions; *RLV*, Rauscher strain of MuLV.

is predicted from the finding of both gs-1 and gs-3 determinants on the same molecule, guinea pig antiserums also contain variable amounts of gs-3 reactivity detected in complement fixation or gel diffusion assays after concentration (*4*).

When the rat and rabbit serums were fractionated on Sephadex G-200, several interesting results were reproducibly obtained (Fig. 1). Unfractionated serums showed a single precipitin line with C-type virus (disrupted by Tween-ether) from hamster (HaLV), cat (FeLV), and rat (RaLV)—an indication of gs-3 antibody. They also gave either one or two precipitin lines with the purified homologous MuLV, regardless of whether the AKR and Rauscher strains of MuLV were used for testing. When concentrated to twice the original volume, the IgM fraction showed no precipitation, while the IgG fraction gave only gs-1 (species-specific) reactions. Surprisingly, reconstitution of IgM and IgG fractions to the original serum volume resulted in a recovery of the gs-3 reactivity. Similar results were obtained with highly purified MuLV and FeLV gs proteins (Fig. 2a) in place of unfractionated viral antigens. Attempts to reconstitute activity with IgM and IgG fractions from normal serums were completely negative.

A further indication that the IgM fraction was essential for gs-3 reactivity was obtained by treating serums with β-mercaptoethanol. This treatment (Fig. 2b) completely eliminated the gs-3 reactivity of both rat and rabbit antiserums.

These data show a novel synergistic effect of IgM- and IgG-containing fractions for demonstration of a cross-reactive antigen in gel diffusion. To our knowledge, a similar phenomenon has not previously been reported for a single serum; however, Goudie *et al.* (*12*) have reported interactions between nonprecipitating and precipitating serums in the case of Hashimoto's disease leading to increased amounts of precipitate and occasionally two precipitin lines with a single antigen. While both the rat and rabbit antiserum fractions showed this synergistic effect, we have tested a goat antiserum to FeLV which contains precipitating gs-3 antibodies in the IgG fraction (*13*). These differences are presumably based on extent of immunization and bleeding schedules.

The erratic observation of one or two precipitin lines between the rat and rabbit antiserums to gs-1, gs-3, and highly purified homologous antigen could be explained on the basis of the differential diffusion rates of the IgM-requiring gs-3 reaction and the IgM-independent gs-1 reaction. That heterogeneity of the antigen is not responsible for the double line could be shown by absorption experiments in which specific gs-1 serum was able to inhibit the precipitin reactions of serums containing gs-3 antibodies. Further, extensive evidence for the presence of both gs-1 and gs-3 determinants in the same structure has been presented, and continued attempts to physically separate the two reactivities have not been successful. While the gs-3 reaction could be explained in the case of reactions

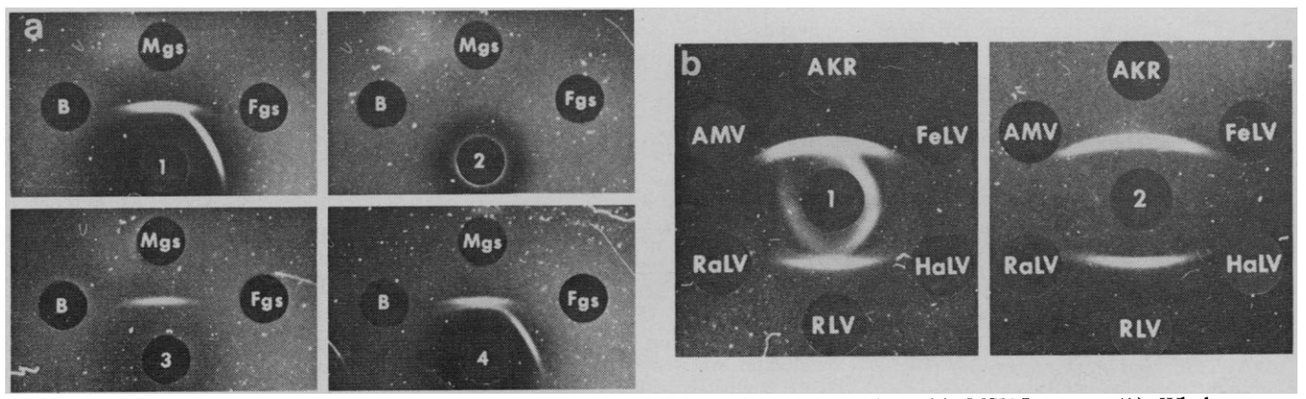

Fig. 2. (a) Gel diffusion patterns of purified murine (*Mgs*) and feline (*Fgs*) gs proteins with MSV-I serum. (1) Whole serum, (2) fraction that contains IgM, (3) fraction that contains IgG, (4) mixture (1:1) of IgM and IgG fractions. (b) Elimination of the gs-3 reactivity of MSV-I serum by treatment with β-mercaptoethanol (see text). Peripheral wells: Tween-ether disrupted C-type viruses as indicated. Center wells: (1) untreated whole serum, (2) β-mercaptoethanol–treated whole serum.

with the homologous antigen by IgM addition to soluble complexes of gs-1 antibody and antigen in antibody excess if we assumed that there was perhaps only one gs-3 site per antigen molecule, this cannot explain the heterologous gs-3 reaction. Here we must assume that both fractions have specific combining ability with antigen. Whether or not each fraction is specific for a distinct site or both have the same specificity can be decided by cross-absorption experiments.

STEPHEN OROSZLAN
DAVID BOVA
ROBERT TONI, RAYMOND V. GILDEN
Flow Laboratories,
Rockville, Maryland 20852

References and Notes

1. A. Gregoriades and L. J. Old, *Virology* **37**, 189 (1969).
2. G. Geering, T. Aoki, L. J. Old, *Nature* **226**, 265 (1970).
3. R. V. Gilden, S. Oroszlan, R. J. Huebner, *Nature New Biol.* **231**, 107 (1971).
4. S. Oroszlan, R. J. Huebner, R. V. Gilden, *Proc. Nat. Acad. Sci. U.S.* **68**, 901 (1971).
5. R. V. Gilden, D. Bova, S. Oroszlan, *Fifth International Symposium for Comparative Leukemia Virus Research*, Padua, Italy, 1971 (Karger, Basel, in press).
6. R. V. Gilden and S. Oroszlan, *Proc. Nat. Acad. Sci. U.S.*, in press.
7. S. Oroszlan, C. L. Fisher, T. B. Stanley, R. V. Gilden, *J. Gen. Virol.* **8**, 1 (1970).
8. S. Oroszlan, C. Foreman, G. Kelloff, R. V. Gilden, *Virology* **43**, 665 (1971).
9. P. Flodin and P. Killander, *Biochim. Biophys. Acta* **63**, 403 (1962); P. Fireman, W. E. Vannier, H. C. Goodman, *Proc. Soc. Exp. Biol. Med.* **115**, 845 (1964).
10. B. Hampar, A. L. Notkins, M. Mage, M. A. Keehn, *J. Immunol.* **100**, 586 (1968).
11. R. J. Huebner, W. P. Rowe, H. C. Turner, W. T. Lane, *Proc. Nat. Acad. Sci. U.S.* **50**, 379 (1963).
12. R. B. Goudie, J. R. Anderson, K. C. Gray, *Immunology* **4**, 309 (1959).
13. Dr. W. Parks, National Cancer Institute, has provided us with a goat antiserum to FeLV which in his laboratory was found to contain gs-3 antibodies in the IgG fraction. We have confirmed this observation.
14. Supported by contract NIH-NCI-E-71-2097 from the Special Virus Cancer Program, National Cancer Institute, Bethesda, Maryland.

15 December 1971; revised 18 February 1972

Isolation and Characterization of Two Major Urinary Metabolites of Δ^1-Tetrahydrocannabinol

Abstract. *Two of the major metabolites which appear in rabbit urine after the administration of Δ^1-tetrahydrocannabinol have been isolated and their structures have been tentatively established. The available evidence indicates that they are 7-carboxy-Δ^1-tetrahydrocannabinols with an additional hydroxyl group on the side chain. The substances occur both free and as conjugates.*

While a good deal is known about the metabolism in vitro of the important cannabinoids (*1–4*), relatively little data has been reported on the metabolic fates of these substances in vivo (*5–7*). We have shown (*8*) that Δ^6-tetrahydrocannabinol (THC) injected into rabbits gives rise to a very small amount of 7-hydroxy-Δ^6-THC in the urine. Lemberger *et al.* (*7*) reported results which suggest that 7-hydroxy-Δ^1-THC occurs in the urine and feces of man after the administration of Δ^1-THC. Many of the above-mentioned studies also showed that most of the THC is metabolized to rather polar materials, with no unchanged drug being excreted. In addition, Agurell and his co-workers (*5*) observed that a large proportion of the urinary metabolites in the rabbit were acidic in nature. We have succeeded in isolating and identifying two of the principal metabolites of Δ^1-THC and now report the results of our work.

We administered ^{14}C-Δ^1-THC (*9*) to adult female New Zealand white rabbits by either a subcutaneous or intravenous route in propylene glycol–water mixtures. The animals were kept in metabolic cages, and urine was collected and frozen every 24 hours for a suitable period. The urine was pooled and acidified to *p*H 3.5. The radioactivity was extracted from the pooled samples via a column of XAD-2 resin (*10*). This treatment allowed the metabolites to be obtained in a concentrated form, free of inorganic salts and other highly polar materials.

The extract was then partitioned between water and chloroform. Only about 20 percent of the radioactivity was found in the organic phase; this chloroform-soluble material was further fractionated by chromatography on a Sephadex G-15 column, and the radioactivity was eluted in a broad band. Most of the nonradioactive material was concentrated in the early fractions. The later fractions, comprising about one-half of the radioactivity, were then combined and fractionated on a DEAE (diethylaminoethyl)-Sephadex column (Fig. 1). The major peak (fractions 150 to 165) was then methylated (*11*) and investigated further.

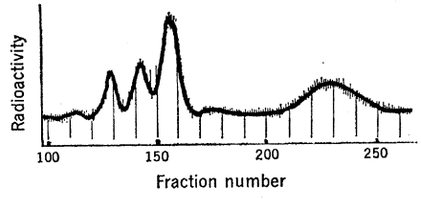

Fig. 1. The elution pattern of the free metabolites when chromatographed on a DEAE-Sephadex column with a NaCl gradient. The radioactivity of the column effluent was monitored by liquid scintillation counting with a Packard flow cell containing anthracene.

A final purification by thin-layer chromatography gave two barely separated zones which were located by radioautography. Most of the radioactivity was contained in these two areas, and their specific activities were close to that of the Δ^1-THC injected into the rabbits. Low-resolution mass spectrometry gave a molecular ion peak at 388 for both metabolites (*12*). This suggests isomeric methyl ester–methyl ether derivatives with a formula of $C_{23}H_{32}O_5$. The principal ions in the high mass end of the spectra of the methylated substances were: metabolite **1** [*m/e* 388 (relative intensity 58), 373 (53), 370 (17), 355 (21), 329 (100), 316 (12), 311 (21)] and metabolite **2** [388 (46), 373 (35), 370 (5), 329 (61), 316 (100), 311 (2)].

The water-soluble metabolites remaining after the chloroform partitioning were next isolated by the following procedure. Alkaline hydrolysis was used to liberate the metabolite from what appeared to be a conjugate with an amino acid. We have not determined the optimum conditions for this reaction, but 2 hours in a refluxing methanol–0.2*N* NaOH solution led to extensive hydrolysis. The hydrolysis mixture was then acidified to *p*H 3.5 and extracted with chloroform; 56 percent of the radioactivity could now be removed from the aqueous phase. The chloroform extract was then partitioned with dilute sodium bicarbonate to separate acidic materials. About 82 percent of the radioactivity was found in the bicarbonate solution, which was then acidified and chloroform extracted. All of the radioactive material soluble in bicarbonate was found in the chloroform phase. These partitioning steps resulted in a large increase in specific activity, so that after methylation (*11*) thin-layer chromatography could be utilized for the final purification. As