Immunological Studies of Y Protein

A MAJOR CYTOPLASMIC ORGANIC ANION-BINDING PROTEIN IN RAT LIVER

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ABSTRACT An antibody produced against rat Y protein, the major cytoplasmic organic anion-binding protein in liver, was characterized. The antibody precipitated Y protein from liver supernatant fractions and specifically removed the organic anion-binding capacity from this fraction.

Y protein was detected by immunodiffusion with this antibody in the supernates of rat liver, kidney, and small intestinal mucosa and was not detected in supernates of 16 other tissues including bile and serum. Precipitation with anti-Y was not detected with supernates of liver from 20 other species, including man.

Quantitative radial immunodiffusion revealed Y protein to constitute 4.5% of supernatant protein in rat liver and approximately 2% of supernatant protein in rat kidney and small intestinal mucosa.

Phenobarbital administration increased the concentration of Y protein in rat liver by 280%, but not in kidney or small intestinal mucosa, and was associated with increased plasma disappearance of sulfobromphthalein sodium, indocyanine green, and bilirubin, and increased hepatic, but not kidney or small intestinal mucosal, content of these organic anions. These observations provide further evidence indicating that the concentration of Y protein is a major determinant of organic anion flux across the plasma membrane of the liver cell.

Immunodiffusion and immunoelectrophoresis revealed serological identity between Y protein, cortisol metabolite-binding protein I, and the major azocarcinogen-binding protein.

INTRODUCTION

Rat hepatocytes contain two cytoplasmic proteins, designated Y and Z, which bind various organic anions including bilirubin, dyes (BSP and ICG),1 drugs, and cholecystographic agents (1, 2). Y protein is considered to be the major binding protein of the two because its hepatic concentration and affinity for various organic anions, whether added in vitro or administered in vivo, is greater than Z protein (1). We have proposed that Y and Z proteins may be involved in the selective transfer of several organic anions from plasma into the liver (1–9). This hypothesis is based upon the following evidence. (a) the Y protein concentration in liver is the highest of all tissues studied (1–3), (b) the direct demonstration of in vitro binding of various organic anions by Y and Z proteins and hepatic uptake in vivo of organic anions by these proteins (1), (c) there is a direct correlation between rates of development of Y protein in neonatal liver and selective hepatic uptake of organic anions after birth (4–6), (d) evidence obtained from phylogenetic studies revealed absence of Y and Z proteins and impaired selective hepatic uptake of organic anions in gill-breathing animals, and the appearance of these proteins with selective hepatic organic anion uptake in air-breathing

1 Abbreviations used in this paper: BSP, sulfobromphthalein sodium; ICG, indocyanine green; SPB, sodium phosphate buffer, 0.01 M, pH 7.4; TEAE, triethylaminoethylcellulose.

The Journal of Clinical Investigation Volume 51 1972 677
animals (7), \( e \) phenobarbital and other drugs, which enhance hepatic organic anion uptake, increase the hepatic concentration of Y protein (2, 8, 9).

In previous studies, Y protein was measured by BSP-binding of liver supernatant fractions using Sephadex G-75 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) chromatography (1-9). Although this technique is quantitative under defined conditions, only qualitative comparisons are valid with columns of different sizes and different amounts of protein (1). Y protein has also been quantitated on polyacrylamide gels after purification from liver supernates (9). This method depends partially on staining characteristics of the protein and standardization is cumbersome, variable, and not suited to multiple measurements (9).

In the present study, a monospecific antibody to Y protein has been produced, and a method for immunologic quantitation has been standardized. This method has been utilized to determine (a) tissue and species specificity of Y protein; (b) whether organic anion-binding by Y fraction in liver supernates results from a single Y protein; (c) the effect of phenobarbital on the concentration of Y protein in relationship to organic anion uptake by various tissues, and (d) identity between Y protein, cortisol metabolite binder I (10), and azocarcinogen-binding protein (11).

**METHODS**

Supernatant fractions were prepared from homogenates of liver and 16 other tissues from 32 adult Sprague-Dawley, 2 Long-Evans, and 2 Gunn (glucurononyltransferase-deficient) (12) rats of either sex. Supernatant fractions were also obtained from at least two specimens of liver from Hartley guinea pigs, albino mice, Rhesus monkeys, Shetland ponies, chickens, rabbits, four species of fish, five species of amphibia, and four species of reptiles. Histologically normal human liver was obtained within 4 hr after patients died from acute myocardial infarction. All laboratory animals were anesthetized with ether and killed immediately; fishes, reptiles, and amphibia were anesthetized with urethane; and horses were killed by captive-bolt after curare administration. Various tissues were rapidly removed, perfused, where possible, with ice-cold 0.9% saline, and a 25% homogenate was prepared in 0.25 M sucrose-0.01 M phosphate buffer, pH 7.4, using a motor-driven, teflon pestle, glass homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.). Kidney and muscle required initial disruption in a VirTis (The VirTis Co., Gardiner, N. Y.) homogenizer. Small intestine was removed from duodenum to terminal ileum, washed with cold 0.9% saline, opened lengthwise, and the mucosa was scraped between two glass slides. Blood was collected and permitted to clot at room temperature; sera were stored at \(-20^\circ\text{C}\).

Bile, obtained from the cunnulated common bile duct of lightly ether-anesthetized Sprague-Dawley rats, was centrifuged at 2500 \(g\) for 30 min. The clear supernate was passed through a Sephadex G-25 column, equilibrated with SPB, to remove bile acids (13). The protein eluted in the void volume was concentrated by ultrafiltration to 1.5 mg protein/ml.

Tissue homogenates were centrifuged at 110,000 \(g\) for 2 hr in a model L Spinco (Beckman Instruments, Inc., Fullerton, Calif.) ultracentrifuge at 4°C. The supernatant fraction was removed and either used immediately or stored at \(-20^\circ\text{C}\).

Two male and two female Sprague-Dawley rats (150-200 g body weight) received daily injections of 8 mg phenobarbital/100 g for 7 days. Four controls received daily subcutaneous injections of saline. All rats received Purina rat chow (Ralston Purina Co., St. Louis, Mo.) and water ad lib. The animal quarters were not sprayed with insecticides to avoid the effect upon hepatic metabolism induced by these compounds.

Y fraction was prepared by Sephadex G-75 chromatography of 110,000 g supernate of homogenates of 18 g of liver from three adult male Sprague-Dawley rats (1).

Y protein was isolated by TEAE anion exchange chromatography and gel filtration on Sephadex G-75 (14-15). The purity of Y protein was established by demonstrating a single homogeneous component using electrophoresis, acrylamide gel electrophoresis, analytical ultracentrifugation, and amino acid analysis. Y protein was stored in SPB at \(20^\circ\text{C}\).

Male domestic American goats, 4-6 months of age, were used for antiserum production. Preimmunization sera showed no precipitation reactions with liver supernate or purified Y protein. Y protein (50-75 \(\mu\)g) dissolved in 1.0 ml SPB, 1.5 ml complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and 10 mg of Mycobacterium butyricum were homogenized for 2.5 min at top speed in a Sorvall (Ivan Sorvall, Inc, Norwalk, Conn.) omnihomogenizer (micro attachment) at room temperature. The emulsion was injected intracutaneously in 0.1 ml amounts at multiple sites. 3 wk later, additional multiple intracutaneous injections were performed with 50 \(\mu\)g of Y protein and incomplete Freund's adjuvant. Antisera were harvested from 500-mI weekly bleedings when a precipitation reaction occurred with a 1:8 dilution of serum and Y protein (100 \(\mu\)g/ml) by Ouchterlony immunodiffusion analysis. When the level of precipitating antibody declined, a final immunization with 50 \(\mu\)g Y protein and incomplete Freund's adjuvant was performed. Blood was allowed to clot at room temperature for 1 hr and then placed at \(4^\circ\text{C}\) for 24 hr after which the serum was removed and centrifuged in a Sorvall centrifuge (Ivan Sorvall, Inc, Norwalk, Conn.).

Male Sprague-Dawley rats received curare. Blood and immune sera were stored at \(-20^\circ\text{C}\) with sodium azide 1:1000 added as a preservative.

Rabbit anti-rat albumin serum was prepared after subcutaneous injection of rat albumin (50 \(\mu\)g) and complete Freund's adjuvant (1 ml) in SPB (1 ml). The injections were repeated 3 wk later. Specificity of this serum was tested by immunodiffusion and immunoelectrophoresis.

**Immunologic methods.** IgG globulin was prepared from immune and normal sera by ammonium sulfate (40%) precipitation followed by DEAE-cellulose anion exchange chromatography (16). The proteins were concentrated to 20.4 mg/ml by ultrafiltration. The concentration of anti-Y IgG was determined from precipitation curves according to Kabat (17). Double immunodiffusion was performed using 1.2% agarose (18), 0.15 M sodium chloride, and 0.05 M sodium phosphate, pH 7.4 with 1:1000 sodium azide. Immunodiffusion plates were incubated at room temperature in a moisture box for 48 hr and at \(4^\circ\text{C}\) for 24 hr. The threshold of detection for this system was 50 \(\mu\)g/ml of purified Y protein.

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Immunoelectrophoresis was performed using 7.5 × 5 cm glass slides coated with 2 mm 1% agarose in Veronal buffer (Winthrop Laboratories, New York) (0.025 μ) pH 8.6. The buffer reservoir contained Veronal buffer, 0.075 μ pH 8.6 and the electrophoresis was carried out at 4–6 volts/cm for 2–3 hr at 4°C (18). After addition of antisera, the slides were examined for precipitin bands 48 hr later. Immunodiffusion and immunoelectrophoresis slides were stained with amido black (19). Quantitative radial immunodiffusion was performed according to Mancini (20). In all studies, 8 ml (±1%) of sample was placed in each well using a Hamilton (Hamilton Co., Whittier, Calif.) micro-syringe. These plates were kept at room temperature in a moisture chamber for 48 hr, photographed at a standard magnification, and precipitation rings were carefully cut out and weighed. Each tissue assay was performed in duplicate at 1:4 and 1:8 dilution of supernates of 25% homogenates. Each plate contained at least three standard concentrations of Y protein in duplicate ranging from 75 to 400 μg/ml. The Y protein content of tissue samples was quantitated by comparison with a standard curve prepared for each plate. Results were expressed as μg of Y protein/mg tissue supernatant protein.

Protein was quantitated (21) using twice crystallized bovine serum albumin as a standard. The E°1cm 280 μm for IgG globulin was taken as 14 (22).

BSP was measured spectrophotometrically at 580 μm after alkalination and its binding was calculated by triangulation (1).

Purified cortisol metabolite-binding protein 1 was supplied by Dr. Gerald Litwack of Temple University School of Medicine, Philadelphia, Pa. Purified azocarcinogen-binding protein was supplied by Dr. Brian Ketterer of the Middlesex Hospital Medical School, London, England. Each purified binding protein was dialyzed against SPB and concentrated to 400 μg/ml and studied by immunodiffusion and immunoelectrophoresis.

Z protein was partially purified from rat liver supernate using Sephadex G-75 gel filtration (1).

Further study of the function of Y protein was performed using anti-Y serum. Three groups of four male Sprague-Dawley rats (150–200 g) received 8 mg phenobarbital subcutaneously daily for 7 days. Three groups of four similar rats served as controls and received daily injections of isotonic saline. The following methods were used to study the effect of phenobarbital on organic anion uptake by liver, kidney, and small intestinal mucosa: (a) Initial disappearance rates from plasma after single intravenous injections of BSP (5 mg/100 g), ICG (1 mg/100 g), and bilirubin (1 mg/100 g), expressed as t½ in minutes, were determined from four to seven carefully timed plasma samples obtained from 2 to 8 min after organic anion administration (9, 23, 24). (b) 3 hr after intravenous administration of BSP, ICG, or bilirubin to rats which were lightly anesthetized with ether and whose body temperatures were maintained at 37–38°C with a heating lamp, laparotomy was performed and, in less than 1 min, the liver, kidneys, and small intestine were removed and weighed. Supernates of homogenates of liver, kidney, and small intestinal mucosa were prepared as described in Methods. For measurement of BSP and ICG in tissues, a 10% homogenate was prepared in distilled water and 1 ml saturated toluidine blue was mixed per 1 ml of homogenate (9). BSP and ICG were quantitated spectrophotometrically after removal of precipitated proteins (9).

Recovery of BSP or ICG added to rat liver, kidney, or small intestinal mucosal homogenates in the range found in various experiments ranged from 72 to 93%. Phenobarbital added in excess to tissue homogenates or plasma did not interfere with chemical estimation of BSP or ICG. In several control and phenobarbital-treated rats, bile duct and duodenal contents were examined visually during laparotomy for ICG and for BSP after alkalination with NaOH; little or no dye was present. A small terminal sample of the liver perfusion was tested and trace amounts of dye were present but were not quantitated. Bilirubin content of liver, kidney, and small intestinal mucosa was determined after preparation of a 25% homogenate in MacIlwain's buffer using the method of Hargreaves (25). Recovery of bilirubin added to homogenates of the three tissues in the range found in liver ranged from 65 to 88%.

RESULTS

Serologic monospecificity of anti-Y antisera was demonstrated by immunodiffusion and immunoelectrophoresis (Figs. 1 and 2). Immunodiffusion revealed a single identical line of precipitation between the purified Y protein and tissue supernates from Sprague-Dawley, Long-Evans, and Gunn rat livers. Immunoelectrophoresis revealed that Y protein migrated toward the cathode. A single precipitin arc was formed with Y protein and liver supernates from the three strains of rats. After absorption with Y protein (2.5 mg/ml), the IgG failed to produce further precipitation with the purified protein or liver supernate. To exclude nonspecific co-precipitation, a nonrelated antigen-antibody system was utilized. Immunodiffusion was performed with rabbit anti-rat albumin immune serum in the center well. Peripheral wells contained 120 mg purified Y or Z proteins/ml rat serum, and supernates of homogenates of rat liver, kidney, and small intestinal mucosa. No precipitation was detected with Y or Z; however, albumin was detected in serum and supernates of liver and kidney.

A quantitative precipitation curve is shown in Fig. 3. The equivalency point for this antisera was 5.1 μg Y/mg IgG. At Y protein concentrations beyond the
equivocally point, slight inhibition was observed which probably reflects limited solubility of anti-Y complexes formed in the presence of excess antigen.

*Effect of anti-Y IgG upon organic anion-binding by "Y" fraction.* Further evidence for the monospecific reaction of the anti-Y IgG with the dye-binding of the "Y" fraction obtained by Sephadex G-75 chromatography was shown by the following experiment. Y fraction (2.9 ml) was added to separate tubes containing 8.7, 5.8, 2.9, and 1.5 ml of anti-Y IgG. Controls included both normal IgG (5.8 ml) added to 2.9 ml Y fraction and Y fraction alone. All volumes were made equal by addition of SPB. The mixtures were made at room temperature for 1 hr and 48 hr at 4°C. The precipitate was removed by centrifugation at 18,000 g for 30 min. BSP (2.74 μmoles) was added to each supernate and the mixture fractionated as previously described. Fig. 4 shows that addition of normal IgG to Y fraction did not reduce BSP-binding by this fraction. No significant reduction of BSP-binding was observed with Y fraction alone. In contrast, addition of increasing amounts of anti-Y IgG resulted in progressive loss of BSP-binding by Y fraction. Gel electrophoresis of the supernatant fractions with no detectable BSP-binding revealed that Y was no longer present after precipitation by anti-Y IgG.

*Tissue and species distribution of Y protein.* Immunodiffusion and immunoelectrophoresis demonstrated the presence of Y protein in liver, kidney, and small intestinal mucosa. A representative immunodiffusion experiment is shown in Fig. 5. Not shown was the observation that the Y proteins of kidney and small intestine had identical electrophoretic mobility as hepatic Y protein. Immunodiffusion analysis was capable of detecting Y protein in a concentration of 3 μg/mg supernatant protein. If Y protein is present in other tissues, its concentration must be less than this amount. Anti-Y IgG did not form identifiable precipitin bands with liver supernates, Y fraction or purified Y protein from other

![Figure 2: Immuno-electrophoresis demonstrates a single precipitin arc with Y protein in the top and third wells, and supernates of Sprague-Dawley liver in second well from top, and Gunn liver in the bottom well. Supernates were undiluted. Cathode is to the right.](image)

![Figure 3: Results of quantitative precipitin reaction of representative goat gamma globulin to rat Y protein. Assay was carried out in duplicate and folin-phenol determination of protein read at OD 660.](image)

![Figure 4: Inhibition of the BSP-binding by anti-Y IgG is demonstrated. BSP-binding by Y fraction (5.72 mg/ml), ○---○; BSP-binding by Y + anti-Y IgG (21 mg/ml) ■—■, and BSP-binding by Y and normal IgG (24 mg/ml) ●—●.](image)

![Figure 5: Immunodiffusion shows cross-reactivity between Y protein and supernates of rat liver, kidney, and small intestinal mucosa. Anti-Y IgG is in the center well. Y protein concentration was 120 μg/ml and supernatant fractions were diluted as labeled.](image)
animal species including man, although organic anion-binding by Y fraction from liver supernates of all vertebrates studied was demonstrable using gel filtration (7). Z fraction failed to reveal precipitation bands when examined by immunodiffusion using anti-Y IgG.

Quantitation of Y protein by radial immunodiffusion and the effect of phenobarbital. Quantitation of Y protein by radial immunodiffusion was reproducible within the range of 75-400 μg/ml. Representative results are shown in Table I. The concentration of Y protein in supernates of liver, kidney, and small intestinal mucosal homogenates from control and phenobarbital-treated rats is shown in Table II. In control rats, Y protein constitutes 4.46 ± 0.5% (σE) of liver, 2.24 ± 0.3% of kidney, and 1.7 ± 0.2% of small intestinal mucosal supernatant proteins. As also shown in Tables II and III, phenobarbital administration increased the concentration of Y protein approximately 280% in liver; the concentrations of Y proteins in kidney and small intestinal mucosa were unaffected. Plasma t½ for BSP, ICG, and bilirubin were reduced by 30-40%, and the hepatic, but not kidney or small intestinal mucosal, content of these three organic anions increased by approximately 40%.

Immunologic relationship between Y protein and other hepatic anion-binding proteins. Immunodiffusion revealed an identical line of precipitation between Y protein, the cortisol metabolite-binding protein I, and the azocarcinogen-binding protein (Fig. 6). Immunoelectrophoresis revealed that the three proteins moved identically toward the cathode and formed single precipitin arcs with anti-Y IgG.

DISCUSSION
The monospecificity of anti-Y is supported by the finding of single precipitin bands with Y protein and liver supernates using immunodiffusion and immunoelectrophoresis. In addition, absorption of the antiserum with Y protein removed all detectable anti-Y reactivity.

The binding of various organic anions to Y protein is electrostatic and, therefore, the respective anion is displaced from the protein during purification by ion-exchange chromatography and gel electrophoresis (1, 15). As a result, sequential BSP-binding by Y protein during various steps in its isolation was utilized as a guide to purification. Proof that organic anion-binding of the Y fraction is due to a single protein is provided by the observation that anti-Y specifically removed virtually all detectable organic anion-binding.

Studies of ontogeny, phylogeny, pharmacologic response, and inhibition of organic anion-binding (1-9) have been performed utilizing liver, and resulted in the hypothesis that Y protein is an important determinant of the relatively selective transfer of various organic anions from plasma into the liver. BSP is largely found in liver after intravenous administration; however, approximately 30% of injected dye is unaccounted for in liver or plasma during the first 5 min after injection (9, 23). Significant amounts of BSP are found in homogenates of kidney and small intestine under these circumstances (26). It was, therefore, of interest to observe that Y protein was detectable in small intestine and kidney by immunodiffusion. Previous studies of

| TABLE I |
| Representitive Reproducibility of Y Protein Quantitation by Radial Immunodiffusion on a Single Plate* |
| Y protein | Disc weight | Mean ±SE |
| μg/ml | μg | mg |
| 311 | 17.2 | 16.9 ±0.3 |
| 155 | 9.8 | 9.7 ±0.2 |
| 78 | 6.0 | 6.4 ±0.2 |

*See text for details.

| TABLE II |
| Concentration of Y Protein in Supernates of Homogenates of Liver, Kidney, and Small Intestinal Mucosa from Control and Phenobarbital-Treated Rats |
| No. | Control | No. | Phenobarbital-treated | P |
| Rats | Liver | Kidney | Small intestinal mucosa | |
| 10 | 44.7 ±2.1 | 14 | 124.0 ±8.9 | <0.001 |
| 8 | 22.4 ±2.7 | 4 | 18.7 ±3.7 | No difference |
| 9 | 17.2 ±1.9 | 4 | 16.7 ±2.9 | No difference |

Results are expressed as μg Y/mg supernatant protein ±SEM. Probability values are based upon comparison of results in control and drug-treated animals.

Hepatic Organic Anion-Binding Protein 681
tissue distribution of Y protein were based upon a standardized BSP-binding system using Sephadex G-75, and Y protein was identified only in liver. The failure to detect Y protein in kidney and small intestine by this system may be explained by the greater sensitivity of the immunologic methods (approximately 500-fold more sensitive than the dye-binding method).

Phenobarbital administration increased the concentration of Y protein in liver as determined by dye-binding (8, 9, 14). Immunologic measurements indicate that Y protein increases approximately 280% thereby excluding a drug-related alteration in affinity of Y protein for the dye (Table II). Studies of radioactive amino acid incorporation indicate that the increased concentration of Y protein observed after phenobarbital administration results from enhanced synthesis (i.e., induction) of Y protein (14). In the present study, phenobarbital administration did not increase Y protein concentration in supernates of kidney or small intestinal mucosal homogenates (Table II), probably because the drug does not become accessible to the site of Y protein synthesis in these tissues. The plasma disappearance rates (t½) for BSP and ICG, as shown previously (9), and bilirubin were increased by approximately 40% after drug administration (Table III). This change was associated with comparable increase in hepatic content of each of the three organic anions; however, their concentrations in kidney and small intestinal mucosa were unaffected. These observations provide further support for the concept that the number of intracellular binding sites provided by Y protein constitute an important determinant of the net flux of organic anions across the plasma membrane.

Morey and Litwack (10) isolated two proteins from rat liver cytosol which bind cortisol metabolites. One of these, designated cortisol Metabolite-binding protein I, was utilized in the present studies. After administration of 4-dimethylaminoazobenzene to rats, threeazo-dye-binding proteins were identified in the soluble supernatant fraction of rat liver (11). The major azo-carcinogen-binding protein (mol wt 45,000) was utilized in the present studies. Immunologic identity was observed between Y protein, cortisol metabolite-binding protein I, and the major azo-dye-binding protein. In other studies, molecular weight, isoelectric point, amino acid analysis, and organic anion-binding characteristics between the three proteins were identical (3, 15). The binding protein, for which the name "ligandin" has

\begin{table}
\centering
\caption{The Effect of Phenobarbital on Plasma Disappearance Rate and BSP, ICG, and Bilirubin Content of Supernates of Homogenates of Liver, Kidney, and Small Intestinal Mucosa}
\begin{tabular}{|c|c|c|c|c|}
\hline
& & Organic anion content & & \\
& & & & \\
& & Plasma disappearance rate & & \\
& & $t_\frac{1}{2}$ min & & \\
& & & & \\
Control (4) & BSP & 3.8 ±0.2 & Liver & 1.84 ±0.21 & 2.05 ±0.32 & 1.76 ±0.34 \\
& ICG & 3.6 ±1.1 & Kidney & 0.032 ±0.011 & 0.041 ±0.010 & 0.035 ±0.012 \\
& Bilirubin & 3.3 ±0.8 & Small intestine & 0.010 ±0.006 & 0.005 ±0.002 & 0 \\
Phenobarbital-treated (4) & BSP & 2.2 ±0.4* & Liver & 2.63 ±0.21* & 2.72 ±0.002* & 2.42 ±0.31* \\
& ICG & 2.1 ±0.2‡ & Kidney & 0.031 ±0.006§ & 0.039 ±0.012§ & 0.029 ±0.008§ \\
& Bilirubin & 2.0 ±0.6* & Small intestine & 0.009 ±0.005§ & 0.002 ±0.002§ & 0§ \\
\hline
\end{tabular}
\end{table}

Results are expressed as mean ± SEM. Probability values are based upon comparison of results in control and drug-treated animals. Numbers in parenthesis refer to number of rats studied.
* $P < 0.01$.
‡ $P < 0.05$.
§ No difference.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure6}
\caption{Immunodiffusion demonstrates immunologic identity between Y protein (120 µg/ml), azocarcinogen-binding protein (Ketterer) (100 µg/ml), normal rat liver supernate (diluted 1:4 with SPB), and cortisol metabolite-binding protein I (Litwack) (100 µg/ml). Anti-Y IgG (20 mg/ml) is in the center well. Peripheral wells are as labeled.}
\end{figure}

682  G. Fleischner, J. Robbins, and I. M. Arias
been proposed (15), binds a range of endogenous and exogenous substances noncovalently with varied affinity (15). These include azo-dye-GSH conjugate (11), corticosterone (27), corticosteroid anionic metabolites (10), estradiol metabolites (27), bilirubin (1), BSP (1), ICG (1), phylloerythrin (2, 14), cholecytostrophic agents (1), flavaspic acid (1), and triiodothyronine. * Carcinogens, such as azo-dyes and methylcholanthrene, bind covalently as well as noncovalently (15, 28). Azo-dye and methylcholanthrene are bound noncovalently as GSH conjugates, and covalently through their cysteinyl residues (28). Similar binding appears to occur after bromobenzene administration (29). These observations suggest several possible functions for the binding protein (15). Noncovalent binding occurs with various organic anions, and the intracellular concentration of Y protein, or, more correctly, the number of available binding sites on Y protein, appears to determine the net flux of various organic anions across the plasma membrane of the liver cell (1-9). We propose that Y protein significantly influences the efflux of various organic anions from the liver cell after their initial transfer across the plasma membrane largely by nonionic diffusion. Whether or not the movement of organic anion conjugates and metabolites from the liver cell into plasma is influenced in a similar manner has not, as yet, been examined. Other compounds, such as certain carcinogens, bind covalently which may protect the cell against carcinogenesis (29, 30) or may be important in promoting carcinogenesis (15, 30). The concentration of the binding protein is very low in hepatomas (15, 31).

The apparent absence of Y protein from the central nervous system may have importance as related to the pathogenesis of bilirubin encephalopathy in newborns (kernicterus). Bilirubin damages mitochondria in neurons in this condition but does not alter hepatic mitochondria in vivo. Y protein may protect hepatic mitochondria but the absence of this protein from neurons may permit injury of neuronal mitochondria to occur.

No data are available regarding the role of Y protein in kidney and small intestinal mucosa. Preliminary immunofluorescent studies indicate that Y protein is predominantly found in parenchymal liver, renal tubular, and intestinal mucosal cells (14). Y protein may play a role in organic anion uptake by these cells; however, this hypothesis has not been examined experimentally.

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684  G. Fleischner, J. Robbins, and I. M. Arias