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Haptoglobin Synthesis. I. *In Vivo* Studies of the Production of Haptoglobin, Fibrinogen, and γ-Globulin by the Canine Liver *

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Haptoglobin is an α_2 plasma glycoprotein that forms a stable complex with hemoglobin (1–5). The complex is cleared from the plasma much more rapidly than free haptoglobin so that marked hypo- or anhaptoglobinemia is a concomitant of hemolysis (6, 7). Since free hemoglobin passes readily into the urine, whereas the complex, perhaps owing to its much greater size, does not, haptoglobin may play a role in the conservation of iron by the body (5, 7). Haptoglobin concentration increases markedly in man (8–12) and animals (13–15) in the presence of inflammation or tissue necrosis. Estrogen administration depresses the haptoglobin concentration in the serum of man (10).

The site of synthesis of this protein is unknown although the liver has been suggested (16), for it is known that in some instances of parenchymatous liver disease haptoglobin concentration is reduced (10, 17). At least theoretically this might be the result of decreased synthesis by a damaged organ. However, increased hemoglobin turnover (18–23) and failure to metabolize estrogens (24) are common features of these cases and could produce the observed hypo- or anhaptoglobinemia. Therefore, this is inconclusive evidence for production of this protein by the liver (10). Possible direct evidence that haptoglobin is synthesized by the liver of the infected mouse was recently presented (25).

The present studies were designed primarily to test the hypothesis that haptoglobin is produced by the liver in the dog. If the hypothesis is correct, there should be no incorporation of administered C^{14}-amino acid into this protein in the hepatectomized animal. Also, under suitable conditions, it should be possible to observe higher specific C^{14} radioactivity of haptoglobin isolated from blood leaving the liver than from that entering it in the intact dog after C^{14}-amino acid is given. To obtain a better understanding of the kinetics of haptoglobin synthesis, it would also be helpful to compare the incorporation curves in plasma with those in lymph. Finally, a comparison of the haptoglobin incorporation curves with the incorporation of the labeled amino acid into fibrinogen, a protein known to be produced by the liver (26–32), and γ-globulin, known to be synthesized chiefly in sites outside the liver (31, 33–38), would provide useful confirmatory information.

Methods

Animals

Apparently healthy mongrel dogs weighing between 16.5 and 22 kg were used. They were maintained on the routine laboratory diet. Each animal received an injection of turpentine (0.5 ml per kg) in multiple subcutaneous sites of the hindquarters while under pentobarbital anesthesia.

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Operative procedures

All procedures were performed under pentobarbital anesthesia with the animals placed on respirators [1] adjusted to a tidal volume of 350 ml. About 65 hours after the turpentine injection, when haptoglobin synthesis is maximal (39), two sorts of operations were carried out. Two dogs (C and D) underwent hepatectomy with the establishment of polyethylene portal-jugular and inferior vena caval-jugular shunts and the placing of a catheter in the left common iliac vein. In four animals, the liver was left *in situ,* and catheters were inserted into the portal and hepatic veins (40) and thoracic duct (dogs F, G, I, and J). After completion of the preparation, all dogs received 250 to 300 μc of glycine-2-C^{14} intravenously over a 10-second period.

Blood and lymph samples

Blood was withdrawn simultaneously from each catheter into syringes containing 3% EDTA in normal saline (1 vol EDTA: 10 vol blood). Collection of each 10-ml sample required 15 to 20 seconds. Blood samples were taken at 10 minutes following isotope administration and then hourly from the two hepatectomized animals for a total of 3 hours and 40 minutes and 6 hours, respectively. Blood samples from the other four dogs were collected at 5- to 10-minute intervals, beginning 10 to 20 minutes after isotope administration, for a total period of up to 1 hour. Lymph was collected continuously into tubes containing 0.5 to 1.0 ml EDTA for 5- to 15-minute periods. Lymph flow was about 0.4 ml per minute.

[1] Harvard Apparatus Co., Dover, Mass.

Protein fractionation

Plasma was obtained from blood, and lymph was freed of cells by centrifugation.

A) Haptoglobin. Three ml of plasma or 1 to 2 ml of lymph was diluted with an equal volume of 0.85% saline. Saturated ammonium sulfate solution (pH 7.0) was added with mixing to a final saturation of 45%. After standing for 2 hours at 4° C, the samples were centrifuged at 105,400 × g for 15 minutes, and the supernatant (45% sup) and precipitate (45% ppt) were separated. Saturated ammonium sulfate solution was added to the 45% sup to a final saturation of 50%, and the mixture was allowed to stand and was centrifuged as outlined above. The precipitate from this step (45 to 50% ppt) was dissolved in a small amount of water and dialyzed exhaustively against 0.85% saline. This material on vertical starch gel electrophoresis and staining with amido black (41, 42) with and without added hemoglobin is shown in Figure 1.

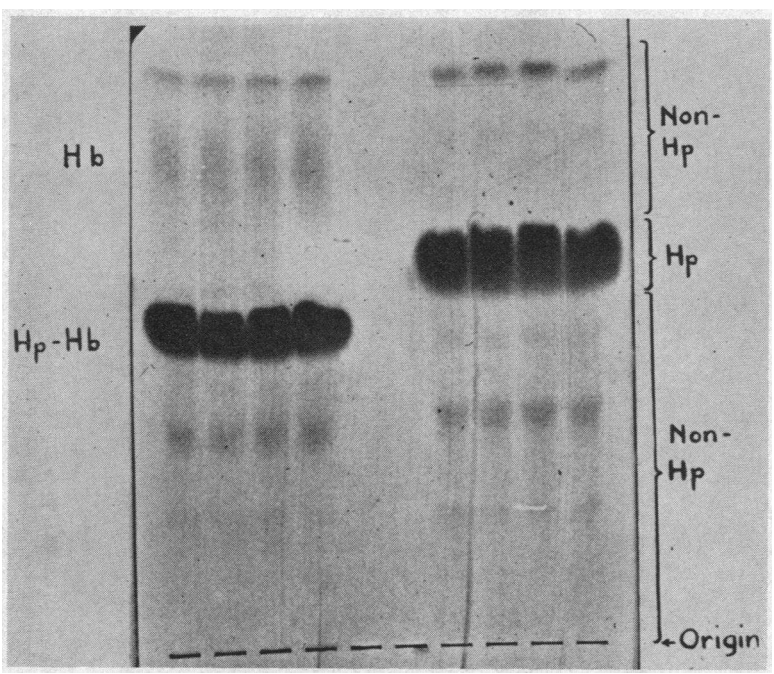

FIG. 1. STARCH GEL ELECTROPHORETIC PATTERNS OF HAPTOGLOBIN PREPARATIONS WITH AND WITHOUT ADDED HEMOGLOBIN. The four patterns to the left show preparations with added human hemoglobin (Hb) in excess of the binding capacity of the haptoglobin. The patterns on the right are the same preparations without hemoglobin. The areas of the stained gels used for determining the percentage of C^{14} radioactivity in haptoglobins (Hp) and nonhaptoglobin (non-Hp) proteins are indicated at the extreme right.

TABLE I

*The percentage of C^{14} radioactivity in haptoglobin in 16 preparations**

Sample no.	% of total radioactivity in haptoglobin†	Sample no.	% of total radioactivity in haptoglobin†
1	88.8	9	90.9
2	88.6	10	89.8
3	89.6	11	91.5
4	90.9	12	89.0
5	92.4	13	93.7
6	88.5	14	92.4
7	89.4	15	92.0
8	94.2	16	91.3

* Obtained from hyperhaptoglobinemic dog plasma from animals who received glycine-2-C^{14} from 1 to 6 hours before sampling.

† See text for method of determining percentage of radioactivity in haptoglobin.

B) Gamma globulin. The 45% ppt from the above fractionation was dissolved in a small amount of water and dialyzed exhaustively against 0.01 M Tris-phosphate buffer pH 8.0 or 0.005 M phosphate buffer pH 6.4 and passed through a column containing 200 to 600 mg DEAE cellulose equilibrated with the same buffer. The columns were washed with this buffer, and the protein eluted was collected and concentrated by dialysis against a concentrated solution of polyethylene glycol 20 M (Carbowax)[2] and then against 0.85% saline. This material on paper electrophoresis (43) contained only diffusively migrating γ-globulin, but on immunoelectrophoresis using rabbit anticanine plasma antiserum two or three minor non-γ contaminants could be seen in some samples.

C) Fibrinogen. Approximately 10 NIH U of bovine thrombin was added with mixing to 1.0-ml samples of the plasma and lymph samples or, in a few instances, to the dialyzed 45% ppt before extraction of γ-globulin. The samples were incubated at 37° C for 2 hours. The clots were wound on glass rods and then washed for 1-hour periods in two changes of 0.85% saline and two changes of deionized water. The washed clots were dried for 48 hours at 73° C.

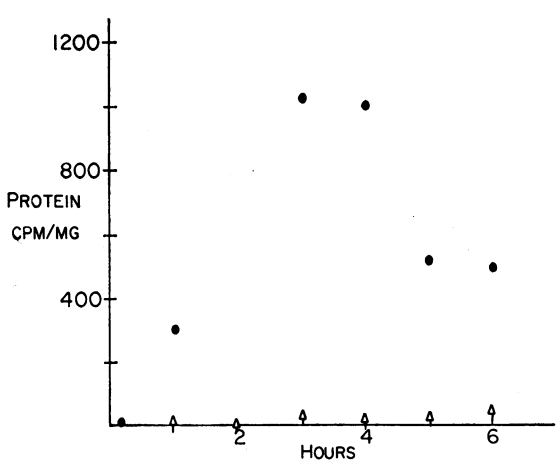

FIG. 2. INCORPORATION OF GLYCINE-2-C^{14} INTO PLASMA HAPTOGLOBIN AND γ-GLOBULIN IN THE HEPATECTOMIZED DOG (EXPERIMENT D). Closed circles (●) indicate γ-globulin-C^{14} specific activity, and open triangles (△) represent haptoglobin-C^{14} specific activity.

[2] Union Carbide Chemical Co., New York, N. Y.

Determination of specific activities

Fifty-μl samples of purified haptoglobin and γ-globulin solution were added directly to the liquid scintillation counting system described by Nathan, Gabuzda, and Gardner (44). Dried clots were weighed, combusted, and counted as described by these authors for hemoglobin. The protein content of the haptoglobin and γ-globulin solutions was determined by the Folin-Ciocalteu method (45) standardized by micro-Kjeldahl analysis (46).

TABLE II

The incorporation of glycine-2-C^{14} radioactivity into plasma haptoglobin and γ-globulin in the hepatectomized dog

Exp.	Hours after glycine-2-C^{14} administered	SA of haptoglobin	SA of γ-globulin
		cpm/mg	*cpm/mg*
C	0.17	1	75
	1.00	2	261
	2.00	9	118
	3.00	13	458
	3.67	11	104
D	0.17	0	5
	1.00	10	307
	2.00	11	
	3.00	22	1,023
	4.00	23	1,004
	5.00	23	515
	6.00	42	512

To demonstrate that the specific activity of the isolated haptoglobin was indeed that of this protein and not disproportionately influenced by some minor contaminant with high specific activity, the following analysis was made. Sixteen haptoglobin samples with high specific activity were subjected to vertical starch gel electrophoresis (41, 42) and stained with amido black. Since no visible bands with the original mobility of haptoglobin appeared after hemoglobin was added (Figure 1), the haptoglobin spot was regarded as containing pure haptoglobin. This spot (Hp-gel) and the gel containing non-haptoglobin protein behind and in front of it (non-Hp-gel) were cut from the stained gel as indicated in Figure 1, dried at 80° C for 48 hours, weighed, and ground to a fine powder with a mortar and pestle. A portion of gel outside the zone of protein migration (blank-gel) was treated similarly. Weighed samples of approximately 50 mg of dried Hp-gel, non-Hp-gel, and blank-gel were combusted and counted as described for fibrinogen.

Blank-gel counts per minute served as background. The mean percentage of radioactivity attributable to haptoglobin in the 16 samples was 90.8 ± 1.8% (1 SD). The actual values are given in Table I.

Results

As Figure 2 illustrates, there is no incorporation of radioactivity into haptoglobin isolated from the plasma of a hepatectomized dog in experiment D. The excellent incorporation of radioactivity into γ-globulin from the plasma of this same animal is also seen in this Figure. The results of experiment C are similar and are presented in Table II.

Figure 3 shows the incorporation of radioactivity into plasma haptoglobin isolated from simultaneous hepatic and portal vein samples over a 1-hour period in experiment F. The haptoglobin

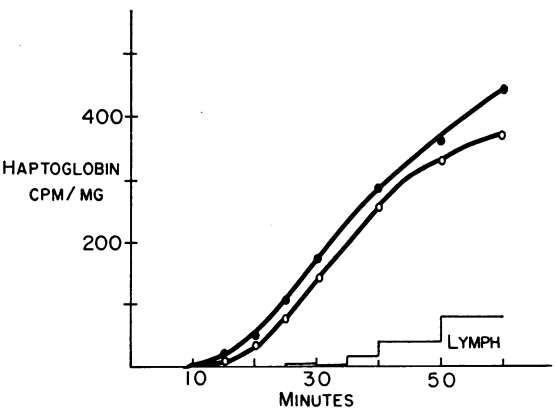

FIG. 3. INCORPORATION OF GLYCINE-2-C^{14} INTO HAPTOGLOBIN ISOLATED FROM HEPATIC VEIN PLASMA (●), PORTAL VEIN PLASMA (○), AND THORACIC DUCT LYMPH (BLOCKS) IN EXPERIMENT F. Lymph flow was fairly constant at about 0.4 ml per minute, and all lymph was collected for extraction of protein.

TABLE III

*The incorporation of glycine-2-C^{14} radioactivity into haptoglobin, fibrinogen, and γ-globulin isolated from portal and hepatic vein plasma and thoracic duct lymph**

Exp.	Minutes after glycine-2-C^{14} administered†	SA of haptoglobin			SA of fibrinogen			SA of γ-globulin		
		PV	HV	TD	PV	HV	TD	PV	HV	TD
		cpm/mg			*cpm/mg*			*cpm/mg*		
F	15 (0–15)	10	18	0	3	7	0	5	4	0
	20 (15–20)	32	48	0	29	42	3	16	18	7
	25 (20–25)	76	103	0	85	139	1	36	38	119
	30 (25–30)	145	172	3	145	183	2	63	82	39
	35 (30–35)	139	185	1	233	258	3	115	83	131
	40 (35–40)	258	287	14	281	305	8	127	119	89
	50 (40–50)	332	361	37	390	414	42	137	152	272
	60 (50–60)	369	447	79	460	495	192	203	208	226
G	15 (0–15)		2	0			18		7	7
	20 (15–20)	24	30	2		19		30	28	25
	25 (20–25)	64	83	0	47	69		50	42	60
	30 (25–30)	131	146	5		134		78	82	59
	45 (30–45)	261	279	47	214	280	42	160	148	151
I	20 (0–20)	57	83	2	64	124	6		19	6
	25 (20–25)	110	138	2		258	1	15	20	10
	30 (25–30)	168	219	3	292		2	35	63	8
	35 (30–35)	266	296	7	441	532	2	23	33	13
	40 (35–40)	316	351	13	537	558	8		35	43
	45 (40–50)	402	409	56	697		45	61	97	101
	52 (50–60)	473	537	150	630	838	182	100	88	127
	60	557	579		742	939		95	70	
J	20 (0–20)	172	226	1	152	191	0	91	81	96
	25 (20–25)	317	383	0	304	403	2	169	121	
	30 (25–30)	501	543	7	485	601	1	209	230	
	35 (30–35)	658	725	19	718	844	39	361	528	
	40 (35–40)	694	749	39	878	939	214	116	336	
	45 (40–50)	731	805	230	931	1,070	823	508	220	
	50 (50–60)	811	903	407	1,101	1,333	1,373	751	1,247	
	60	995	1,073		1,325	1,557		740	918	

* PV = obtained from portal vein plasma, HV = obtained from hepatic vein plasma, and TD = obtained from thoracic duct lymph.

† The numbers to the left outside parentheses refer to time of plasma sampling; those within parentheses indicate the period over which lymph was collected.

specific activity is higher in hepatic vein plasma than portal vein plasma for all points. The results of experiments G, I, and J (Table III) similarly show all hepatic vein haptoglobin specific activities higher than the corresponding portal vein values. Furthermore, haptoglobin isolated from thoracic duct lymph has much lower specific activity at any given time and begins to rise significantly only after some 35 minutes.

Although the absolute specific activities are not comparable, the incorporation curves for fibrinogen in experiment I, shown in Figure 4, are quite similar to those of haptoglobin. They show the same difference in specific activity of fibrinogen isolated from hepatic and portal plasma and the same slow and delayed rise in lymph specific activity. Experiments F, G, and H resulted in curves similar to experiment I (Table III).

By contrast, γ-globulin specific activities in this same experiment (Figure 5) show no consistent difference between the veins (although the experimental variation in points is greater), and the appearance of radioactivity in the lymph is prompt and of about the same magnitude as that in the plasma. The γ-globulin and fibrinogen curves for experiments F, G, and J (Table III) are essentially the same as in experiment I.

Discussion

The failure of the hepatectomized dog to synthesize haptoglobin as well as the finding that haptoglobin specific activity is higher in hepatic vein than portal vein plasma indicates that in the dog this protein is produced by the liver. These data do not exclude other sites of synthesis but make them unlikely. Since selective catabolism of nonradioactive haptoglobin molecules would appear to be very improbable, the inflow-outflow difference in specific activities across the liver most probably represents incorporation of radioactive amino acid into newly synthesized protein in this organ. This reasoning was the basis of the classical work of Miller, Bly, and Bale (36, 37) concerning the site of synthesis of the plasma proteins in the rat. They found little incorporation of radioactive amino acid into α-globulins in the hepatectomized animal, whereas incorporation into α-globulins was excellent in their isolated, perfused rat liver preparation. Kukral and associates (31) obtained somewhat different results; they found that approximately one-third the normal amount of labeled amino acid was incorporated into α-globulins by the hepatectomized dog. Whatever these α-globulins are, these experiments indicate that haptoglobin is not one of them. These authors also observed, as we have, excellent incorporation of radioactivity into γ-globulin by the hepatectomized animal.

Williams, Asofsky, and Thorbecke (25) studied the incorporation of radioactive amino acid into plasma proteins by *in vitro* incubation of organ slices from infected mice. By using radioimmunoelectrophoresis they were able to show up-

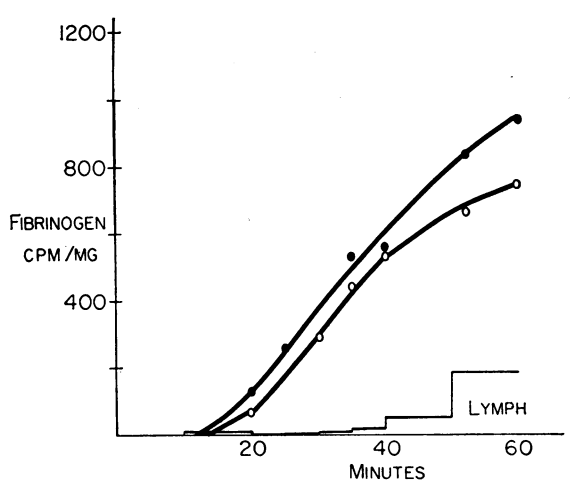

FIG. 4. INCORPORATION OF GLYCINE-2-C^{14} INTO FIBRINOGEN ISOLATED FROM HEPATIC VEIN PLASMA (●), PORTAL VEIN PLASMA (○), AND THORACIC DUCT LYMPH (BLOCKS) IN EXPERIMENT I.

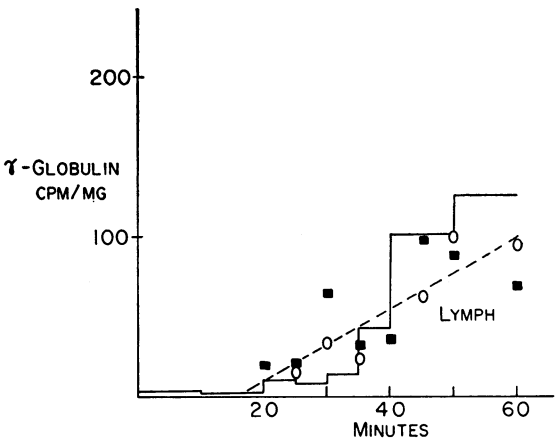

FIG. 5. INCORPORATION OF GLYCINE-2-C^{14} INTO γ-GLOBULIN ISOLATED FROM HEPATIC VEIN PLASMA (■), PORTAL VEIN PLASMA (○), AND THORACIC DUCT LYMPH (BLOCKS) IN EXPERIMENT I.

take of radioactivity into haptoglobin-hemoglobin complexes in incubation media from liver and possibly also spleen preparations but not from other tissues. The interpretation of these data is made difficult because mouse spleen is hematopoietic (47) and mouse blood has a rather high reticulocyte level (48), so that radioactivity may have been incorporated into hemoglobin and hence into hemoglobin-haptoglobin complexes, if hemoglobin from hemolysis of newly formed erythrocytes were released into the medium.

In all the present studies, haptoglobin behaved like fibrinogen, our model for a protein produced in the liver. Since both haptoglobin and fibrinogen appeared in the lymph well after the liver to thoracic duct transit time (about 8 to 14 minutes)[3] and specific activities rose only slowly, it would appear that release of protein from the site of synthesis in the liver is directly into the blood rather than into the lymph and thence to the blood. The prompt appearance and rapid rise of γ-globulin specific activity in the lymph speaks for the release of at least part of the newly synthesized protein directly into the lymph from sites outside the liver, as one might expect from the observations of Asofsky and Thorbecke (49).

It has been suggested that the rise in haptoglobin concentration in the serum following inflammation might be due to release of the protein from a preformed pool (11). Evidence refuting this hypothesis has also been presented (15). The present data suggest that the rise in serum haptoglobin level following inflammation is the result of increased synthesis with prompt appearance of newly synthesized molecules in the circulation.

Summary

The incorporation of glycine-2-C^{14} into plasma haptoglobin, fibrinogen, and γ-globulin is studied in two hepatectomized and four intact dogs during response to inflammation. There is incorporation of radioactivity into γ-globulin but not into haptoglobin in the hepatectomized animals. The specific activity of haptoglobin isolated from hepatic vein plasma is higher than that isolated from simultaneously obtained portal vein plasma at all times up to 1 hour in the four intact dogs. This difference in specific activity is also observed in the case of fibrinogen. There is no difference in specific activity between γ-globulin isolated from portal vein plasma and that obtained simultaneously from hepatic vein plasma. Labeled haptoglobin and fibrinogen appear in thoracic duct lymph only after approximately 35 minutes, and specific activities are considerably lower than corresponding specific activities in the plasma throughout the period of study. In contrast, labeled γ-globulin appears in the lymph within 10 minutes, and specific activities are comparable to those in the plasma. These results provide evidence that haptoglobin is synthesized by the canine liver. They also indicate that newly synthesized haptoglobin and fibrinogen molecules enter the plasma directly, whereas γ-globulin is at least partially released directly into the lymph.

Addendum

A report (50) appeared after the preparation of this manuscript that describes observations of serum haptoglobin types in a case of human liver transplantation. The donor had type 2-2 haptoglobin, whereas the recipient's was type 2-1. On the second day post-transplantation, the recipient had type 2-2 haptoglobin in her serum, but no haptoglobin was demonstrable after this time. The patient received 7,000 ml of blood during and after liver transplantation. The authors present these observations as evidence for production of haptoglobin by the human liver.

We have observed a similar situation in which a liver from a donor with haptoglobin type 2-1 was transplanted into a recipient with type 2-2 haptoglobin. The recipient was transfused with 16,000 ml of blood. Haptoglobin was demonstrable from the time of transplantation to death 11 days postoperatively, and at all times the haptoglobin was of mixed types, reflecting the mixture of types in the transfused blood.

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[3] The liver to thoracic duct transit time was determined in these experiments by injecting a solution of Evans blue dye into the substance of the liver and noting the time of its first appearance in the lymph.

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