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Further Purification of a Human Placental Inhibitor to Hemagglutination by H-1 Virus* (32665)

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A previous communication (1) described the preliminary purification of a substance, observed by Toolan (2) in all human placental fluids, that inhibits hemagglutination by H-1 and HB viruses, but not hemagglutination by the closely related RV or H-3 viruses; this material is not present either in maternal or fetal sera. It was learned that the inhibitor is an immunologically homogeneous glycoprotein that produces a single band on paper electrophoresis. Although a number of glycoproteins have been found that inhibit hemagglutination by influenza virus (1), the only two which have been successfully purified to date are urinary mucoprotein (3) and a group of inhibitory glycoproteins found in the submaxillary glands of ruminants (4). In both instances gentle chemical procedures were employed to obtain the final fractions. More drastic methods appear to break down the glycoprotein molecule. Since one of the steps previously reported in the method used for purification of the H-1 hemagglutination inhibitor involved precipitation with sulfosalicylic acid, it seemed possible to us that some denaturation with breakage of the native molecule into smaller units might have occurred during this procedure. Further work was done, therefore, to develop a simple and gentle technique for isolating the placental inhibitor in a pure form. The present paper describes such a method, together with several physical and chemical properties of the inhibitor.

Materials and Methods. Fluid was obtained from fresh unwashed placentas by removing the fetal membranes and allowing liquids of the placental mass to leak out into the containing vessel. Most of the placentas were delivery-room specimens handled with asceptic precautions. A few were obtained under sterile conditions at caesarian section. Approximately 20–75 ml of fluid were collected from each full-term placenta and spun for 15 min at 2,000 rpm to separate the red blood cells. Samples (0.3 ml) of the supernatant fluids

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were taken for haptoglobin typing and the remainder was frozen at —20°C. The placental fluids were separated into 2-2, 2-1, and 1-1 types according to the heme-haptoglobin patterns. Only the 1-1 fluids were used in the purification since the 2-2 and 2-1 haptoglobins are large molecules that tended to contaminate the final fractions obtained in the purification procedures. A volume of 200 ml of the 1-1 material was unfrozen and employed for each experiment.

Hemagglutination-inhibition tests. Hemagglutination-inhibition activity was tested as described previously (1).

Chemical analysis. Protein was determined by the Lowry method (5).

Disc electrophoresis. Individual placental fluid proteins were separated by disc electrophoresis in a Canalco model-6[1] apparatus. The procedures employed were those recommended in the Canalco equipment instructions for serum proteins or other samples of similar or more dilute protein content. Volumes of 3, 10, 20, or 40 μl were used, depending on the concentration of the protein solution to be analyzed. The placental fluid fractions were stained with Amido Black, Schiff reagent, or Oil red O (6).

Ultracentrifugation. Centrifugation was carried out in a 30 rotor or a 39 swinging bucket rotor in a Beckman-Spinco L preparative ultracentrifuge. Sucrose gradients were formed by layering 1-ml fractions of 10, 20, 30, and 40% sucrose; 0.35 ml of the material to be tested was then layered on the sucrose and spun at 35,000 rpm in a 39 swinging bucket rotor for 22 hours. Fractions of 5 drops each were collected through the bottom after puncturing the tube. Forty fractions were obtained from each tube.

Immunology. Immunodiffusion in ionagar was performed according to the method described by Ouchterlony (7). The following anti-human globulin sera, all prepared in goats, were obtained from Hyland Laboratories, Los Angeles, California and tested against whole placental fluids and purified fractions: anti-α_2 M macroglobulin, anti-β lipoprotein, anti-ceruloplasmin, anti-haptoglobin, anti-IgA globulin, anti-IgG globulin, anti-transferrin, anti-orosomucoid, and anti-chorionic gonadotropin. Also tested were anti-IgM macroglobulin (goat) from Sylvania Co., Millburn, N. J., and anti-group specific globulin (rabbit) from Lloyd Bros., Inc., Cincinnati, Ohio. Quantitation of α_2-M macroglobulin was done according to a micromethod modification of the immunodiffusion technique of Gell *et al.* (8) described elsewhere (9). Quantitation of IgM was done by the radial-diffusion technique (10); commercially available Hyland Laboratory plates were employed for this test.

Treatment with proteolytic enzymes. Trypsin (2× cryst.), chymotrypsin (3× cryst.), pepsin (2× cryst.) were obtained from Worthington Biochemical Corp., Freehold, N.J. The inhibitor fractions (both those obtained after kaolin extraction and the active materials from the sucrose gradients) were treated with the above enzymes and their inhibtory activity then measured. Each enzyme was added to a final concentration of 1 mg/ml in 0.1 *M* phosphate buffer, pH 7.1. In the case of papain the final solution used was 0.01 *M* in cysteine and disodium ethylenediamine tetraacetic acid. Incubation was carried out for 1 and 2 hours at 37°C. Controls were also incubated and tested for hemagglutination inhibition (HA-I) activity.

Treatment with neuraminidase. Neuraminidase (RDE) enzyme (1800 IU/ml when reconstituted) was obtained from Sigma Chemical Co., St. Louis, Missouri. The purified inhibitor (active fractions from the sucrose gradients) was mixed with an equal amount of enzyme solution made up in phosphate buffer pH 6.3 and incubated 24 hours at 37°C. Controls containing no neuraminidase were incubated under identical conditions. HA-I activity was measured immediately following incubation.

Reaction with periodate. Purified inhibitor was mixed with an equal amount of 0.1 *M* sodium meta periodate in acetate buffer pH 5.1, and allowed to stand at 4°C for 24 hours. The periodate action was stopped by the addition of 1/4 volume 20% sucrose. HA-I activity was then measured in the treated samples and in controls without periodate.

Reaction with 2-mercaptoethanol. Purified

[1] Canalco Indust. Corp., Bethesda, Md.

inhibitor was diluted 1/2 with 0.2*M* mercaptoethanol in phosphate buffer pH 7.1 and incubated 24 hours at 37°C. Controls without 2-mercaptoethanol were also incubated. HA-I activity was then determined.

Effect of sugars on inhibitory activity. One per cent solutions of several sugars in 0.1*M* phosphate buffer pH 7.1 were mixed with an equal amount of H-1 virus for 1 hour at room temperature. The sugars employed were: galactose, mannose, rhamnose, glucosamine, and *N*-acetylglucosamine. HA-I activity was determined by adding 0.4 ml of sugar and virus mixture to each glycoprotein dilution in the test instead of adding H-1 virus plus phosphate-buffered saline (PBS) as was done in the controls. Other controls for this test were sugar plus red cells alone and sugar plus virus with red cells subsequently added.

Reaction with antiserum to α-macroglobulin. Purified fractions [$(NH_4)_2SO_4$ precipitation supernatant fluid and active sucrose-gradient pool] were combined with the gamma fraction from the α_2-M antiserum (prepared by DEAE-cellulose fractionation) in a 1:2 volume ratio for 1 hour. The HA-I titer was then determined. After precipitation with $(NH_4)_2SO_4$ to remove the gamma globulins, the HA-I titer was again determined. The supernatant fluid was tested for the presence of α_2-M macroglobulin by immunodiffusion.

Purification of the inhibitor. Kaolin was used according to the method of Clark and Casals (11) and others (12,13) to absorb nonspecific virus hemagglutination inhibitors from the placental fluids. Pooled placental fluids of 1-1 haptoglobin type were first diluted 1:5 with borate-buffered saline (BBS) pH 9.0 and then added to an equal volume of kaolin prepared as a 25% suspension in BBS. This mixture was allowed to stand 20 min at room temperature and subsequently centrifuged at 2,000 for 15 min. The resulting supernatant fluid called "kaolin-absorbed fluid," was considered to represent a 1:10 dilution of the original placental fluids. Such absorbed fluids were then divided into 30-ml aliquots and spun at 30.000 rpm for 12 hours after which the upper 25 ml in each tube were carefully removed and discarded. From the 5 ml remaining in the tube, approximately 4 ml were added to a pool to be spun once more; the jelly-like pellet plus the overlying 1 ml of supernatant fluid were saved. The pooled 4-ml fractions were centrifuged for 12 hours at 30,000 rpm in tubes containing 30 ml each. The pellets plus the overlying 1 ml of fluid of this second centrifugation were added to the first centrifugation pellets. Then $(NH_4)_2SO_4$ in powder form was added very slowly with constant stirring until a molarity of 1.9 was obtained. This material was centrifuged at 2,000 rpm for 15 min and the supernatant fluid which contained all the inhibitory activity, was then dialyzed for 24 hours against saline after which it was filtered through a column (4 × 60 cm) of Sephadex G-200 equilibrated with Tris buffer, pH 8.0. The active column eluate was concentrated by evaporation and subsequently layered on a sucrose gradient and spun for 22 hours at 35,000 rpm. The active sucrose-gradient fractions were reacted with anti-α_2M gammaglobulin (1:2 by volume) for 1 hour and precipitated with $(NH_4)_2SO_4$ as before. The resulting supernatant fluid contained all the inhibitory activity.

Results. The purification procedure produced a product where more than 99.9% of the initial protein was removed but at least 60% of the original HA-I activity remained (Table I). Immunodiffusion tests indicated that none of the following serum proteins contaminated the final product: IgM globulin, IgG globulin, α_2M macroglobulin, β lipoprotein, ceruloplasmin, haptoglobulin, transferrin, orosomucoid, chorionic gonadotropin, or group-specific proteins.

Kaolin absorption for removal of nonspecific virus inhibitors also removed most of the β lipoprotein. Preparative ultracentrifugation concentrated the inhibitor and separated it from the lighter placental fluid proteins. By quantitative immunodiffusion, it was shown that in the $(NH_4)_2SO_4$ precipitation step, IgM and IgG globulins were separated from the inhibitor. It was also noted that the supernatant fluid of the $(NH_4)_2SO_4$ precipitation where the activity remained, contained only 10 to 15% of the α_2M macroglobulin found in the pellet. Finally, after Sephadex G-200 filtration and sucrose-gradient centrifu-

TABLE I. HA-I Activity of Various Fractions Obtained during Purification of Placental Inhibitor.

	HA-I titer	Total units of HA-I activity	Total protein mg	Purification factor
Original placental fluid	640[a]	12.8×10^{4}[b]	12,600.0	1.0
Kaolin-absorbed fluid	80	12.1×10^{4}	6,666.0	1.8
Centrifugation pellet	2,560	16.6×10^{4}	2,990.0	5.8
$(NH_4)_2 SO_4$ supernatant fluid	2,560	16.6×10^{4}	436.0	39.0
Active column eluate	5.120	14.3×10^{4}	28.0	515.0
Active sucrose fractions	10,240	10.2×10^{4}	13.8	515.0
Pure glycoprotein after reaction with Anti-α_2M gamma globulin	5,120	10.2×10^{4}	5.6	1750.0

[a] Guinea pig cells were employed for these procedures. Results are expressed as the highest dilution that completely inhibited hemagglutination.

[b] Titer times number of ml of material

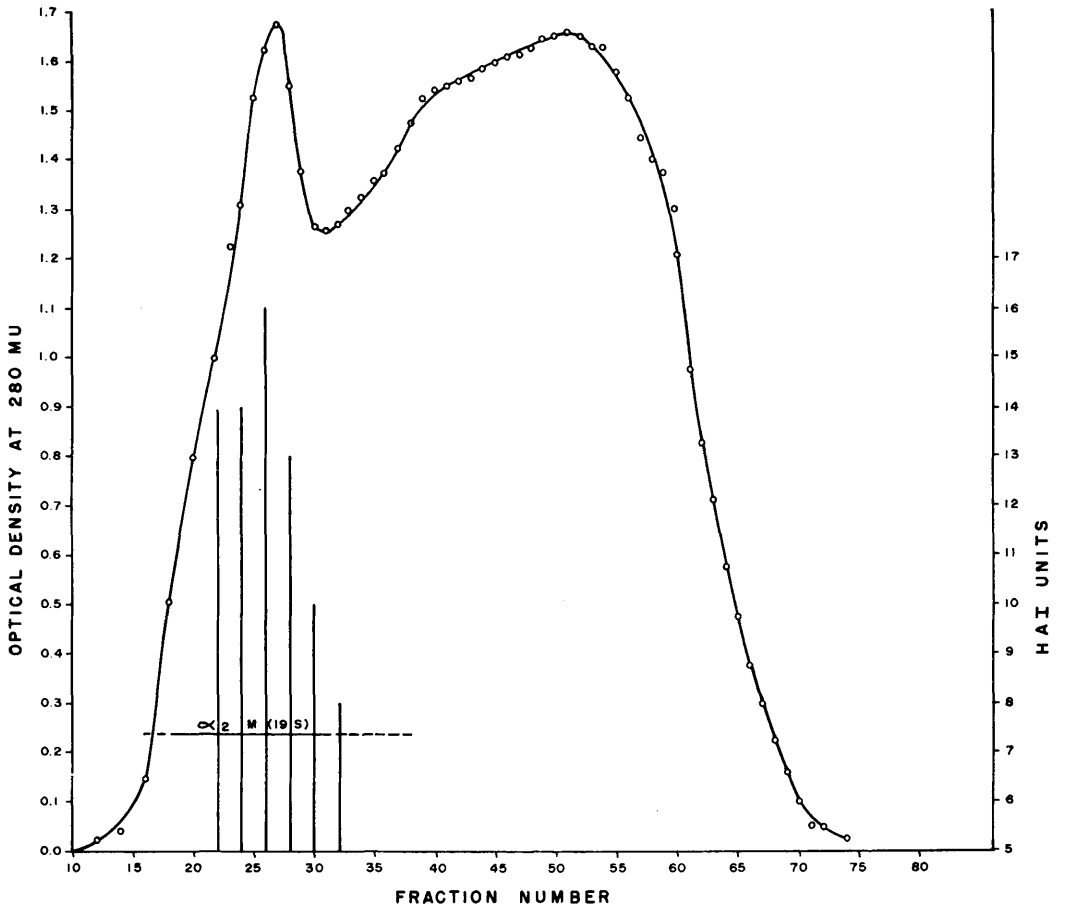

FIG. 1. Elution diagram of Sephadex G-200 column fractions. The solid horizontal line indicates the alpha α_2M distribution as determined by diffusion in agar gel. Trace amounts are shown by broken lines. The vertical solid lines represent hemagglutination-inhibition activity of the various fractions.

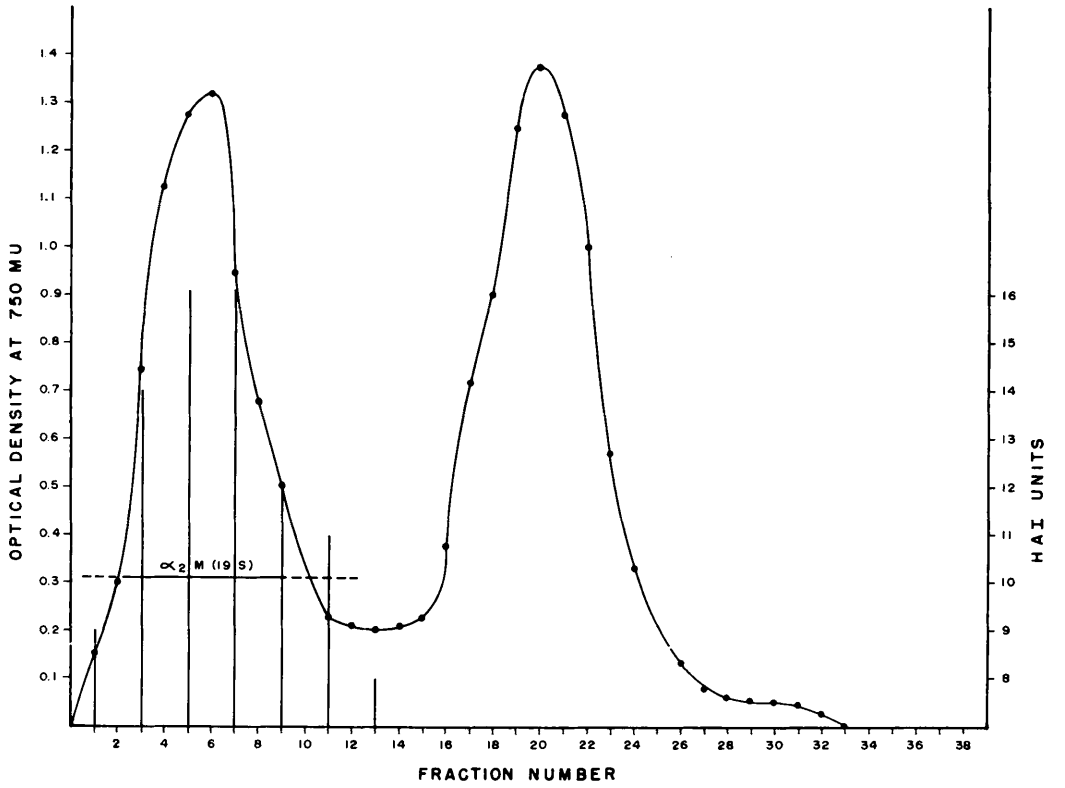

FIG. 2. Sucrose density gradient diagram of active Sephadex G-200 column fractions. Refer to legend Fig. 1 for further details.

gation, a fraction was obtained which contained only macroglobulins, namely α_2M macroglobulin and the placental inhibitor.

As can be seen in Fig. 1, the inhibitory activity appeared in the first peak eluted from the Sephadex G-200 column, indicating that the inhibitor was a large molecule (14). When the active fractions 18–31 were then centrifuged on a sucrose gradient, the activity appeared in fractions 2–11 close to the bottom of the tube (Fig. 2). This is the same area as that where α_2M macroglobulin, a 19S serum protein, is found. However, it was apparent that the inhibitor was not the usual serum α_2M macroglobulin since no HA-I activity was lost after reaction with anti-human α_2M gamma globulin. After precipitation with $(NH_4)_2SO_4$ to remove the gamma-globulin, the inhibitory activity still remained in the supernatant fluid. When this supernatant fluid was tested by immunodiffusion for the presence of α_2M macroglobulin, negative results were obtained. Attempts to separate the α_2M macroglobulin and the placental inhibitor by stepwise elution from a DEAE-Sephadex column (15) were unsuccessful. Only 25% of the inhibitor activity could be recovered with phosphate buffer 0.4*M*, pH 5.2.

Fractions obtained at various steps of the purification procedures were also examined by disc electrophoresis. Patterns of the kaolin-absorbed fluid and the active sucrose-gradient fractions, after staining with Amido Black, are illustrated in Fig. 3 The sucrose-gradient fractions show only one line corresponding to the α_2M impurity and the inhibitor itself. Staining with Oil red O gave negative results. Staining with Schiff reagent also gave one line as might be expected from the carbohydrate content of both α_2M and the inhibitor.

In our previous work (1) with placental fluids, it was observed that the inhibitory activity was very stable. Neither heat, pH,

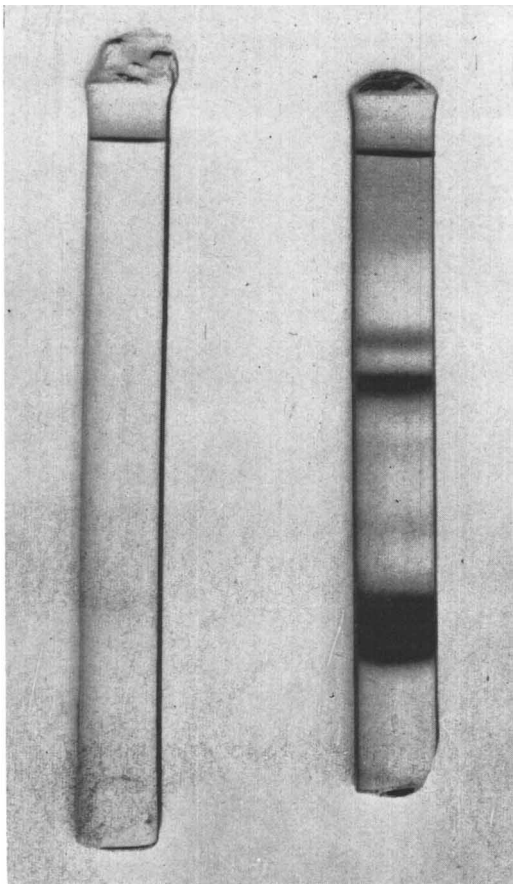

FIG. 3. Disc-electrophoresis protein patterns of the kaolin-absorbed placental fluid and also of the active sucrose-gradient fractions.

concentration, or dialysis altered the inhibitory titer. It is known also that placental fluids can be stored for a year at 4°C without loss of HA-I activity (16). However, in the present study it was found that the active Sephadex-column eluate and the sucrose-gradient pool began to lose activity after a few weeks at 4°C. After removal of the α_2M macroglobulin impurity, the very dilute active fraction remaining was unstable and occasionally lost its titer in as short a time as a few hours.

The inhibitory activity (Table II) was destroyed by 1-hour treatment with trypsin, chymotrypsin and papain. On the other hand, 1-hour treatment with pepsin had no effect. Treatment with neuraminidase left less than 1%of the activity, and sodium metaperiodate completely inactivated the placental inhibitor, an indication of its glycoprotein nature. *N*-acetyl glucosamine, rhamnose, mannose, and galactose interfered with the reaction of inhibitor and virus as shown by loss in HA-I titer. Finally, treatment with mercaptoethanol did not destroy the HA-I activity.

Discussion. In previous work (1) the human placental inhibitor was purified by using sulfosalicylic acid to precipitate out contaminating proteins. The material obtained appeared to be immunologically and electrophoretically homogeneous. However, examination of the purified inhibitor in the analytical ultracentrifuge (17) showed a great amount of heterogeneity. A sedimentation coefficient analysis gave a value of 7.8S for the faster peak in the solution. Such heterogeneity could be explained on the basis of polymerization of a single species. The glycoprotein, isolated by Tamm and Horsfall from urine, for example, has four molecular species, all breakdown products of the intact glycoprotein; the molecular weights of each have been determined (18). DiFerrante and Popenoe (19) isolated a protein which Maxfield (20) demonstrated was one-half of the urinary glycoprotein. Maxfield (21) also showed that the intact glycoprotein of Tamm and Horsfall readily produced quarter molecules if the

TABLE II. Sensitivity of the Hemagglutination-Inhibitory Activity to Various Treatments.

Treatment	Hemagglutination-inhibitory activity	
	Before	After
Trypsin	640[a]	0
Chymotrypsin	640	20
Papain	640	0
Pepsin	640	640
Neuraminidase	2560	160
$NaIO_4$	320	0
2-Mercaptoethanol	320	320
Mannose	1280	0
N-Acetyl glucosamine	1280	320
Rhamnose	1280	160
Galactose	1280	160
Glucosamine	1280	1280
α_2M antiserum	1280	1280

[a] See footnote Table I.

purification procedure of DiFerrante and Popenoe was employed. It could be further dissociated by means of urea, alkali, acid, heat or nonaqueous solvents. Preparation of the smaller fragments have not been made in pure form. It appears, therefore, that in the isolation of large glycoproteins, mild methods of purification should be used to avoid breakage of the native molecule.

In the present study, a simple and gentle method was employed for isolating the H-1 placental inhibitor. In our previous work (1) an alternative procedure that did not include treatment with acid resulted in a product that was contaminated with haptoglobins and α_2M macroglobulin. To avoid the haptoglobin contamination in the present study, only placental fluids containing 1-1 haptoglobins were used. Since 1-1 haptoglobin is a small molecule even after reaction with hemoglobin, it could be almost totally eliminated by Sephadex G-200 filtration and completely eliminated after sucrose-gradient centrifugation. By reaction with anti α_2M gammaglobulin and subsequent further purification by ammonium sulfate precipitation or sucrose-gradient centrifugation, the α_2M contamination was prevented.

The placental inhibitor sedimented as a macroglobulin by density-gradient ultracentrifugation. By disc electrophoresis and immunodiffusion it behaved as a homogeneous product. Its activity was destroyed by trypsin, chymotrypsin, and papain indicating its protein nature. The fact that mercaptoethanol did not affect the inhibitory activity indicated that the purified substance was not an IgM; this finding was confirmed by the absence of IgM in the immunodiffusion tests. Lack of activity after neuraminidase or periodate treatment indicated not only the glycoprotein nature of the inhibitor but also the likelihood that sugar groups in the molecule are involved in the reaction with H-1 virus. This latter finding is also supported by the inhibition of the placental glycoprotein action when it is tested in the presence of rhamnose, galactose, mannose, and *N*-acetylglucosamine.

It is of interest that the human inhibitor occurs in placental fluids only, and not in sera or urines (2–16). This finding rules out any relationship of the unknown substance to placental lactogen which is produced by the placenta but is present in pregnancy sera and urine as well (22). Chorionic gonadotropin, a second placental hormone, is, like placental lactogen, a small protein (30,000 mw); it is also present in pregnancy sera as well as urine (23). The placental inhibitor thus appears to be a third protein product of the placenta.

Summary. The glycoprotein found in human placental fluids that inhibits the hemagglutination of H-1 and HB viruses has been obtained in a high degree of purity as shown by disc electrophoresis and immunodiffusion. It sediments as a macroglobulin whose biological activity can be destroyed by treatment with trypsin, chymotrypsin, papain, neuraminidase, or sodium metaperiodate. The inhibitor possesses a remarkable capacity for reacting with H-1 virus since it is active in as low a concentration as 0.004 μg/ml.

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Malaria: Extracellular Amino Acid Requirements for *in Vitro* Growth of Erythrocytic Forms of *Plasmodium knowlesi** (32666)

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Previous investigations showed that *dl*-methionine was essential for both *in vivo* and *in vitro* growth and multiplication of erythrocytic forms of *Plasmodium knowlesi* (3,4). The growth medium of those *in vitro* experiments contained monkey serum which could supply other amino acids required for plasmodial growth. In the present paper the amino acid requirements of *P. knowlesi* were investigated further by studying plasmodial growth in media depleted of various amino acids.

Materials and Methods. The preparation of plasmodial cultures, described previously (5), was modified as follows:

Parasites. Blood was obtained from Rhesus monkeys (*Macaca mulatta*), infected with *P. knowlesi* by blood passage, when the parasitemia had reached 10–30% rings or trophozoites. The number of parasitized cells was reduced to 10% by mixing with uninfected monkey erythrocytes, when the parasitemia was higher than 10%. The erythrocytes were washed three times in 10 volumes of ice-cold Hanks' salt solution containing 100 mg/100 ml glucose. Each culture contained 11 ml of medium and 0.03 ml packed parasitized erythrocytes. Cultures were incubated at 37°C for 18–20 hours until the majority of the parasites of the control cultures had developed to immature schizonts and a few segmenters. Each culture was made in duplicate and all media were tested for parasitic growth in one experiment, repeated six times.

Medium. Thirteen lots of Eagle's basal medium (1) were made, differing from each other only in that one of the following 13 amino acids was missing: *l*-cystine, *l*-arginine, *l*-tryptophan, *l*-histidine, *l*-leucine, *l*-isoleucine, *l*-lysine, *l*-methionine, *l*-glutamine, *l*-tyrosine, *l*-threonine, *l*-valine, *l*-phenylalanine. A fourteenth medium containing all of the mentioned amino acids served as a control. All media contained 0.2 m*M* of each of the following amino acids: *l*-alanine, *l*-aspartic acid, *l*-glutamic acid, *l*-proline, *l*-serine, and glycine.[1] The media were supplemented with 10% fresh human AB serum, dialysed against saline until free from amino acids as tested by complete disappearance of *l*-leucine-^{14}C, *l*-isoleucine-^{14}C, or *l*-histidine-^{14}C added to the serum prior to dialysis.

Determination of DNA. In a second set of three experiments, 1 μC orotic-6-^{14}C acid[2] hydrate (specific activity 3.88 mC/mmole) was added to each culture at the onset of incubation and DNA was extracted at the

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[1] Nutritional Biochemicals Corp., Cleveland, Ohio and Sigma Chemical Co., St. Louis, Mo.

[2] New England Nuclear Corp., Boston, Mass.