TABLE IV. Minor Components of Calcareous Corpuscles According to Emission Spectroscopic Analysis (Melpar).

	Material from		
	Chile	New Zealand	Lebanon
Aluminum	+	+	+
Boron	—	—	—
Cadmium	+	+	+
Copper	—	—	—
Iron	+	+	+
Lead	—	—	—
Manganese	—	+	+
Nickel	+	+	+
Sodium	+	+	+
Strontium	+	+	+
Tin	—	—	—
Titanium	+	+	+

+ sign indicates that the compound occurs in the corpuscles; — sign that it has not been demonstrated.

nickel, strontium, and titanium which have not been reported from the previously studied species.

Our present findings indicate that the corpuscles isolated from the same species of tapeworm can show differences when worms from different geographic localities are studied. The differences, however, are minor when compared with the very major ones observed between various species. It is thus probable that the latter are essentially species-specific.

Summary. Calcareous corpuscles isolated from larval *Echinococcus granulosus* collected from sheep in Chile and New Zealand and from cattle in Lebanon showed only minor differences in their chemical composition and their heat-induced crystallization patterns. The only definite differences found were that the Chilean material lacked manganese, and that it had a somewhat higher magnesium and a lower phosphorus content than the other samples. As a consequence of the relatively low phosphorus content the Chilean material yielded upon heating at most traces of apatite. Previously described much more pronounced differences in chemical composition and crystallization patterns between corpuscles from different tapeworm species are hence probably essentially species-specific.

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A Human Placental Fluid Inhibitor to Hemagglutination by H-1 and H-B Viruses: I. Purification.* (30544)

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The H-viruses are a group of small agents that produce a specific deformity in newborn hamsters. Two main types are known which are serologically distinct: H-1 virus which was first isolated from a transplantable human tumor(1,2) and subsequently from human placentas, embryos, and neoplasms(3), and RV, isolated from a rat tumor(4). H-3 is related to RV. A third and rare type of H-virus is HB which has only been isolated from a few fresh human placentas and from one fresh human neoplasm(3). It is serologically unlike either H-1 or RV. All these agents agglutinate red cells from a variety of animal species in distinctive patterns which allow diagnosis of their type(3,5). The hemagglutinating ability of H-1 which has been studied(6) has been found to be remarkably stable. Since it had been reported by Toolan that H-1 and HB could be isolated from human placentas, it was of interest when the same author(3) noted that all human placental fluids, even when extracted with Kaolin to remove non-specific inhibitors, still prevented the hemagglutination of H-1 virus with

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any type of reacting red cell but did not affect the hemagglutination of H-3 or RV viruses. An inhibitory effect was also seen on the hemagglutination of HB virus, particularly with guinea pig cells, less so with hamster cells. Extracted normal human sera, urine, amniotic fluids or cord bloods did not affect hemagglutination of any of the viruses. The present paper describes the isolation and characterization of the placental inhibitor.

Methods and materials. Placental fluid. Fluid was expressed from fresh unwashed placentas by incision and pressure with a sterile scalpel. Most of the placentas were delivery room specimens handled with aseptic precautions but a number of placentas were also employed that were obtained under sterile conditions at caesarian section. The experimental results were similar for both groups. Approximately 30 ml of fluid was collected from each full-term placenta and spun for 15 minutes at 2000 rpm to separate the red blood cells. Supernatants of 15-25 placentas were pooled and stored at 4°C until used.

Hemagglutination-inhibition tests. Hemagglutination-inhibition (HA-I) activity was tested by making serial 2-fold dilutions of the test placental fluids or purified materials in 0.2 ml of phosphate-buffered saline (PBS), pH 7.2. To each dilution was added 0.2 ml of virus containing 8 hemagglutinating units. The mixture was allowed to stand one hour at room temperature, after which 0.4 cc of a 0.4% solution of washed guinea pig red blood cells was added and the tests put in a cold room at 4°C overnight to be read next day. The inhibitory activity of the fluid was read as the reciprocal of the highest dilution that completely prevented hemagglutination of the cells.

Chemical analyses. Protein was determined by the Folin-Ciocoteau method(7); hexose by a slight modification of the orcinol method of Lustig and Langer as employed by Weimer and Moshin(8); hexosamine by the procedure based on the Elson-Morgan method as employed by Rimington(8); and sialic acid by the diphenylamine reaction according to the procedure of Winzler(8).

Ultracentrifugation. Ultracentrifugation was performed with a 30 rotor in a Beckman Spinco L preparative ultracentrifuge.

Electrophoresis. A Gelman Electrophoretic Apparatus was employed with the use of Sepraphore III (microporous cellulose polyacetate) paper and a barbital buffer, pH 8.6.

Immunology. Gel diffusion in ionagar was performed according to the method described by Ouchterlony(9). The following anti-human globulin sera, all prepared in goats, were obtained from Hyland Laboratories, Los Angeles, Calif., and tested against normal human sera, whole placental fluids and purified inhibitor: anti-macroglobulin, anti-transferrin, anti-β lipoprotein, anti-ceruloplasmin, anti-orosomucoid, anti-IgM macroglobulin, anti-IgA globulin, anti-IgG globulin, and anti-chorionic gonadotropin. Also tested were: anti-IgM macroglobulin (goat) and anti-IgG globulin (goat) from Sylvania Co., Milburn, N. J. and from Immunology Inc., Lombard, Ill., as well as anti-chorionic gonadotropin (rabbit) and anti-$\alpha 2$ macroglobulin (rabbit) from Immunology, Inc., and anti-IgM macroglobulin, anti-IgG globulin, and anti-IgA globulin, all of rabbit origin, from Lloyd Bros., Inc., Cincinnati, Ohio.

Purification of the inhibitor. Procedure A. The following purification procedure was first used. Pooled placental fluids diluted 1:5 with borate buffered saline (BBS) pH 9, were extracted with an equal volume of Kaolin prepared as a 25% solution in BBS and then centrifuged at 2000 rpm for 15 minutes. This procedure removed any non-specific inhibitors present. The resulting supernatant was centrifuged at 30,000 rpm for 9 hours, and the jelly-like pellet thus obtained was diluted 10-fold and centrifuged again at 30,000 rpm for another 12 hours. To the second centrifugation pellet, which was of similar consistency as the first, plus the overlying 1 ml of supernatant, $(NH_4)_2SO_4$ in powder form was added very slowly with constant stirring until a molarity of 1.9 was obtained. This material was centrifuged at 2000 rpm for 15 minutes and the supernatant which contained the inhibitory activity was filtered through a column (4 $\times$ 60 cm) of the cross-linked Dextran-gel, Sephadex G-200, equilibrated with phosphate buffered saline (PBS), pH 7.2.

TABLE I. Purification of Placental Fluid Inhibitor by Procedure A.

Sample	HA-I titer*	Total units of HA-I activity	Total protein (mg)	Purification factor
Orig. placental fluid	640	9.6×10^4†	12120	1
Kaolin extract‡	80	9.8×10^4	8832	1.4
1st centrifugation pellet§	5120	14.3×10^4	1557	11.3
2nd " " ‖	5120	9.7×10^4	453	26.8
$(NH_4)_2SO_4$ super¶	5120	8.0×10^4	141	72.9
Active column eluate**	2560	9.5×10^4	42	284

* HA-I titers for H-1 virus. Titers against HB virus were comparable. All specimens were tested also for HA-I activity against RV and H-3 and were consistently negative.

† Titer times number of ml of material.

‡ Placental fluid diluted 1:5 with BBS (borate buffered saline) and extracted with equal volumes of Kaolin made up to 25% with BBS.

§ Pellet obtained from centrifugation of the Kaolin extract for 12 hr at 30,000 rpm.

‖ Pellet derived from centrifugation of 1st cent. pellet, diluted 10-fold, for 12 hr at 30,000 rpm.

¶ Supernatant obtained when 2nd cent. pellet was adjusted to 1.9M with $(NH_4)_2SO_4$.

** Sephadex G-200.

Procedure B. It was observed that when placental fluids were treated with an equal amount of 3% sulfosalycilic acid, the supernatants obtained had as much activity as the Kaolin extracted supernatant fluids. This was the basis for the second procedure employed. A powder form of $(NH_4)_2SO_4$ was added to the pooled placental fluids with constant stirring until a concentration of 1.9M was obtained. This step was included to precipitate out IgA globulin which otherwise contaminated the final material. To the supernatant of the $(NH_4)_2SO_4$ precipitation, an equal volume of 3% sulfosalicylic acid was added. Care had to be taken to add this acid slowly and with constant stirring or as much as 50% of the inhibitory activity could be lost as determined by HA-I tests. The supernatant was then first dialyzed against 0.15M NaCl until no more reaction was observed in a spot test for sulfosalicylic acid employing 1% $Fe(NO)_3 \cdot 9\ H_2O$, and secondly, dialyzed against phosphate-buffered saline pH 7.2 overnight. Finally, the dialyzed material was centrifuged for 12 hours at 30,000 rpm in a 30 rotor and the pellet obtained, plus the overlying 2 ml, was filtered through a Sephadex G-200 column (4 × 60 cm), equilibrated with PBS.

Heat and pH studies. Purified fractions from both purification procedures were heated in a water bath at 56°, 70°, 85° and 100°C for one hour. After the materials were cooled to room temperature, the HA-I titer was determined. From purification procedure A, the Kaolin extracted placental fluid and the active concentrated column eluate, diluted 1:8, were studied. From procedure B, since less material was available, only the pellet from the 30,000 rpm centrifugation, diluted 1:8, was employed for the heat studies.

The stability of the inhibitor was checked over a range of pH 1 to 12 using sodium phosphate-citric acid buffer and borate-sodium hydroxide buffer(10). One part of the purified sample, diluted 1:8, and 9 parts of buffer were mixed and stored at room temperature for 1 hour. Equal parts of PBS were added to adjust the samples to the conditions of the hemagglutination assay. Thus only irreversible inactivation was studied. Both the active concentrated eluate, diluted 1:8 with PBS, from procedure A, and the pellet, also diluted 1:8 with PBS, of the 30,000 centrifugation from procedure B, were tested.

Results. The purification and recovery of HA-I activity obtained for the different steps in procedure A is given in Table I and for procedure B in Table II. Fig. 1 is the elution diagram of purified inhibitor from the column of both procedures. The inhibitory activity was found in the first of two peaks. Either purification method produced a product where more than 99% of the initial protein was removed but at least 70% of the original HA-I activity remained.

The column eluate from procedure B was analyzed for its carbohydrate and protein content. Table III gives the results of the chemical determinations which indicate that the

TABLE II. Purification of the Placental Fluid Inhibitor by Procedure B.

Sample	HA-I titer*	Total units of HA-I activity	Total protein (mg)	Purification factor
Orig. placental fluid	640	19.2×10^4	22000	1
$(NH_4)_2SO_4$ super†	640	17.0×10^4	8139	2.3
Sulfosalicylic acid super‡	320	14.6×10^4	296	5.7
Centrifugation pellet§	5120	16.9×10^4	100	190
Active column eluate‖	2560	11.4×10^4	18.2	700

* HA-I titers for H-1 virus. Titers against HB virus were comparable. All specimens were tested also for HA-I activity against RV and H-3 and were consistently negative.
† Supernatant obtained when placental fluid was adjusted to 1.9M with $(NH_4)_2SO_4$.
‡ Supernatant obtained after diluting the $(NH_4)_2SO_4$ super with 3% sulfosalicylic acid.
§ Pellet derived from centrifugation of the sulfosalicylic acid super for 12 hr at 30,000 rpm.
‖ Sephadex G-200.

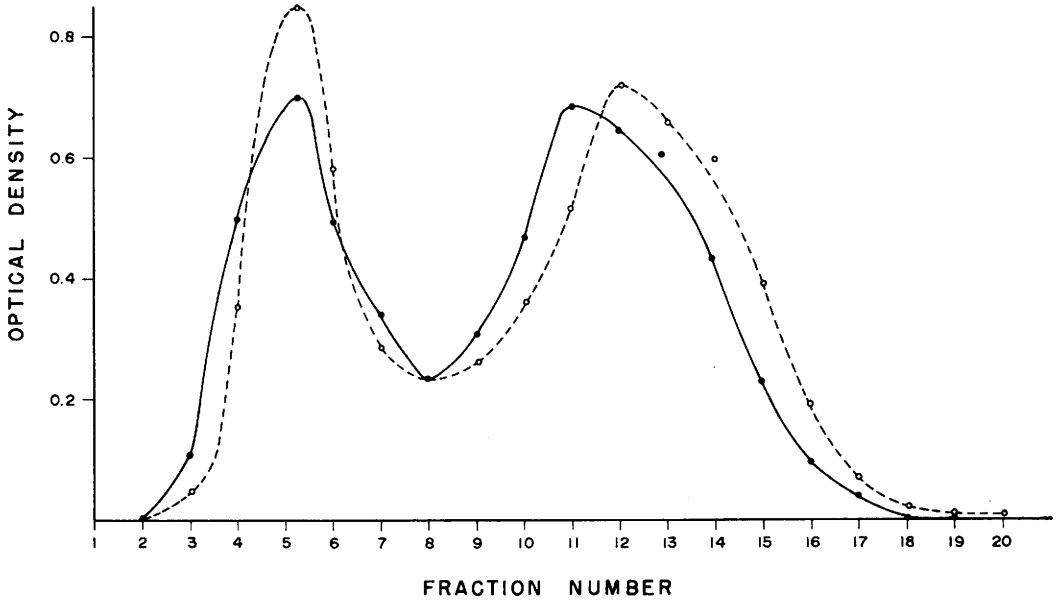

FIG. 1. Elution diagram of column eluates as obtained from procedure A (dotted line) and from procedure B (solid line). All of the inhibitor was in the first of the 2 peaks.

placental fluid inhibitor is a glycoprotein, since it contains hexose, hexosamine and sialic acid, the 3 major elements of a glycoprotein. The ultraviolet absorption spectrum of the inhibitor had an absorption maximum at a wave length of 280 mm showing that no nucleic acid was present. Electrophoresis of the final purified products from the two procedures gave the results shown in Fig. 2. The column eluate from procedure A had two components present as indicated by the 2 lines in the $\alpha 2$ and $\beta 1$ regions while the purified fraction from procedure B appeared to be electrophoretically homogeneous since it had but one line in the slow α or fast β area. In immunodiffusion plates set up with the final column eluate of procedure B, there was no reaction with the following substances all of which reacted both with sera from the placental donors and with unabsorbed† placental fluids: anti-ceruloplasmin, anti-transferrin, anti-orosomucoid, anti-β lipoprotein, anti-IgA globulin, anti-IgM globulin, anti-IgG globulin, anti-

TABLE III. Chemical Composition of Inhibitor from Placental Fluid.

Protein	73–78%
Hexose	9–11%
Hexosamine	7– 8%
Sialic acid	4– 6%

† Absorption with Kaolin removes most of the β lypoprotein, IgM globulin and chorionic gonadotropin from the placental fluids.

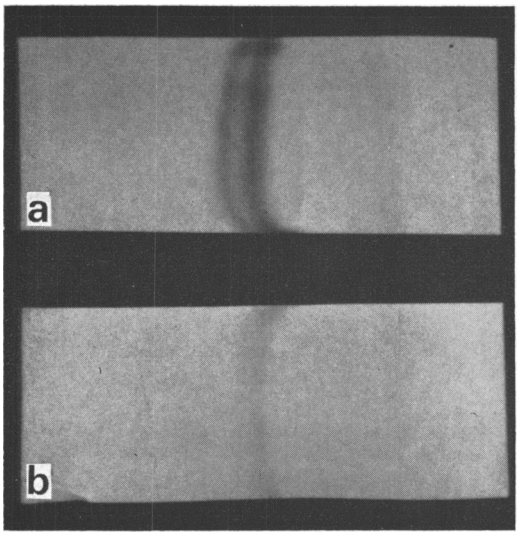

FIG. 2. Electrophoretic patterns of purified inhibitor a) as prepared by procedure A showing 2 bands in α_2 and β_1 regions and b) as prepared by procedure B with only one band in the slow α or fast β area.

$\alpha 2$ macroglobulin, anti-haptoglobulin, or anti-chorionic gonadotropin. A very faint line was seen with anti-transferrin, present as an impurity, since a strong line for transferrin was present in the second peak which came out of the column. This peak contained no inhibitor. The column eluate prepared by procedure A had, in addition to the faint reaction with anti-transferrin, some reaction with anti-$\alpha 2$ macroglobulin and with anti-haptoglobulin. This finding is in accord with the electrophoretic studies which showed two lines with the final fraction of procedure A in contrast to the single line of the eluate from procedure B.

Stability of the inhibitor to variations in heat and pH was remarkable. No loss of HA-I activity occurred even when the inhibitor was heated at 100°C for one hour. This finding is consistent with the glycoprotein nature of this substance since many glycoproteins are known to be very heat stable. Hirst(11) reported that the glycoproteins in human plasma which inhibit hemagglutination by influenza virus could be boiled at a concentration of 1.5% and subsequently show a 16-fold increase in titer; at a concentration of 3%, no change in titer occurred. The fractions studied in the present experiments had a concentration of 0.7%, 0.05% and 0.007%. In all 3 cases no reduction in the HA-I titer was observed. During the purification procedures, a range of pH from 1 (3% sulfosalicylic acid supernatant) to 9 (Kaolin extracted placental fluids) was incurred and did not affect the inhibitory activity. This gave an early indication of the pH stability of the material. Subsequent tests indicated that no change of titer occurred when the purified inhibitor was subjected to a pH range of 1 to 12.

Discussion. Perchloric, sulfosalicylic and trichloroacetic acids have been used by numerous investigators to precipitate out contaminating substances in the purification of glycoproteins(12,13,14). When placental fluids were precipitated with 3% sulfosalicylic acid, the supernatants thus obtained possessed HA-I titers comparable to those found in the Kaolin extracted placental fluids. On the other hand, treatment of the placental fluids with tungstic acid which is known to precipitate glycoprotein left no inhibitory activity in the supernatants. These observations together with the heat stability of the placental extracts gave the first indications that the inhibitor might be a glycoprotein. The chemical properties of the placental fluid inhibitor also indicate that it is a glycoprotein. Purification of this substance can be accomplished without difficulty and purified material can be obtained active to 0.00012 mg/ml.

Several glycoproteins have been found in nature that inhibit hemagglutination by influenza viruses. Stulberg(13) found that a glycoprotein isolated from human plasma by the perchloric acid filtrate method inhibited influenza virus hemagglutination. Similar inhibitors which have been reported are: ovarian cyst mucin(15), ovomucin(16,17), human red blood cell substances(18,19), bovine salivary mucine(20), and urinary mucoprotein (21,22). Of these substances, only urinary mucoprotein is homogeneous. Since glycoproteins isolated by more drastic procedures(23, 24) apparently are of much smaller size than the huge urinary mucoprotein molecule, it is possible that the isolation of glycoproteins in their native state may be, in some instances, as demanding a task as the isolation of certain

other large molecules from biological materials, *e.g.*, nucleic acids in their native state.

Other than neutralizing antibody, no other inhibitor of hemagglutination by the H-viruses than the placental glycoprotein has been reported. It is of interest that the inhibitor occurs in placental fluids only and not in sera or urines(3,25). This finding would appear to rule out any relationship of the unknown substance to placental lactogen which is produced by the placenta but is present in pregnancy sera and urines as well (26). Another placental hormone, chorionic gonadotropin, was missing from the purified fractions as determined by immunodiffusion techniques. The inhibitor thus appears to be a third product of the placenta. Apparently all human placental fluids contain this substance. Among 600 placentas tested to date were a number from embryos; one of special note was the placenta from a 3-months ectopic pregnancy implanted in the fallopian tube (25). The placental fluid from this embryo had a high HA-I titer.

Immunodiffusion techniques indicated that nine of the major serum components were absent in the purified inhibitor and that human chorionic gonadotropin was also missing. Only a trace of transferrin was found. These immunodiffusion studies strongly suggest that the purified fraction might be immunologically homogeneous. Further immunoelectrophoretic work will have to be done to clarify this point.

Summary. A substance found in human placental fluids that inhibits the hemagglutination of H-1 and HB viruses but not that of H-3 or RV viruses, has been purified and found to be an electrophoretically homogeneous glycoprotein stable to heat and a wide pH range. It also appears to be immunologically homogeneous and occurs in the slow α or fast β region.

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