almost complete lack of cholesterol absorption in the pancreatectomized subjects, an evaluation can be made of the significance of the intestinal phase of cholesterol metabolism on the blood cholesterol level. It can be estimated that perhaps as much as 50% or more of the serum cholesterol in man is derived from cholesterol entering the circulation via the intestine. It is also of interest that the liver and other tissues did not have the capacity to maintain the serum cholesterol level. Other lines of evidence (10–12) also indicate that the intestine makes an important contribution to the blood cholesterol pool in man. The ileal and jejunocolic bypass procedures give a pronounced drop in blood cholesterol levels. In one study (12) normal intestinal continuity was restored and serum cholesterol rose from 81 to 275 mg/100 ml.

Summary. Two human subjects with total pancreatectomy showed very little absorption of orally administered cholesterol-4-^{14}C following discontinuance of oral pancreatin. During this period their serum cholesterol dropped to under 100 mg/100 ml. When oral pancreatin was restored to one of the subjects, cholesterol was absorbed and the serum cholesterol level returned to 200 mg/100 ml. The results of the present study indicate that pancreatic secretions are necessary for the proper absorption of cholesterol and the maintenance of the blood cholesterol level in man.

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The Nonspecific Inhibitors of Rubella-Virus Hemagglutination* (33071)

H. J. DOLD[1] AND R. L. NORTHROP (Introduced by Friedrich Deinhardt)
Departments of Microbiology, Presbyterian-St. Luke's Hospital and University of Illinois Medical and Graduate Schools, Chicago, Illinois

Human serum contains nonspecific inhibitors of rubella-virus hemagglutination (1–3) which must be removed before hemagglutination-inhibiting (HI) serum antibodies can be measured. Currently kaolin is used to absorb serum prior to performing the rubella HI test. It is known, however, that kaolin is a nonselective adsorbent of serum proteins (4,5) often reducing the antibody titers of serum.

This communication reports that the nonspecific inhibitors of rubella-virus hemagglutination are removed from human serum by the addition of manganous chloride and heparin, a process which specifically precipitates β-lipoprotein (5–7).

Materials and Methods. Sera. The sera were separated sterily from the blood of infants, children, and young adults and were

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stored at —20°C without prior heat-inactivation.

Antigen preparation. Rubella hemagglutinating (HA) antigen was prepared in tissue cultures of baby hamster kidney (BHK-21) cells according to the method of Stewart *et al.* (1). The Gilchrist strain of rubella virus adapted to the BHK-21 cells was used as the inoculum. Fluids containing HA were treated with Tween 80 (polyoxyethylene sorbitan monooleate) and diethylether (8) to stabilize and to increase the titer of the antigen.

HA test. The microtiter method of titrating HA antigen in this study was a modification of the procedure described by Stewart *et al.* (1) and will be described in detail elsewhere (9). In brief, the modification consisted of the use of phosphate buffer, pH 6.2 containing 0.03% bovine albumin, 0.5% dextrose, 0.03% gelatin, 0.01% calcium chloride and 0.01% magnesium chloride (ADGP buffer) as diluent.

HI test. The HI test was a modification of the method of Stewart *et al.* (1). As in the HA test, the ADGP buffer was used as diluent for serum and antigen.

Serum neutralization. Rubella-virus neutralizing antibody was assayed according to the method described by Parkman *et al.* (10), with the modification that Coxsackie A-9 was used as the challenge virus.

Serum preparation. Kaolin treatment. A 0.1-ml portion of each serum was absorbed with 0.7 ml of a 25% suspension of acid-washed kaolin (Fisher Scientific Co.) in ADGP diluent for 20 min at room temperature. After centrifuging at 1000 rpm for 5 min in a refrigerated centrifuge, 0.05 ml of a 50% suspension of either adult or 1-day-old chicken erythrocytes was added to the supernatant and the mixture was held for 60 min at 4°C. The red cells were sedimented by centrifugation as before.

Manganous chloride–heparin (M-H) treatment. To a 0.2-ml portion of each serum, 0.1 ml of 0.1 *M* manganous chloride ($MnCl_2$) was added. The mixture was shaken and 0.1 ml of heparin (Ries Biologicals, Inc.) diluted with deionized water to contain 500 units/ml was added. The mixture was held for 15 min at 4°C with frequent shaking. Without centrifuging, 0.05 ml of either a 50% suspension of adult, or 1-day-old, chicken erythrocytes was added and the sample was held for an additional 30–60 min at 4°C. The mixture was centrifuged at 1000 rpm for 5 min at 4°C and the supernatant was collected. When the precipitate, which always formed after the addition of $MnCl_2$ and heparin, was to be analyzed the sample was centrifuged as above before the chick cells were added. The precipitate was dissolved in 0.2 ml of 10% sodium citrate solution (5) and the solution was dialyzed overnight against isotonic caline.

Qualitative and quantitative immunoglobulin analyses. Immunoelectrophoresis of serum or serum fractions was performed in a LKB appartus (LKB Products, Inc., Stockholm, Sweden) according to the method of Scheidegger (11) using antisera specific for human IgA, IgG, IgM, and β-lipoprotein (Hyland Laboratories, Los Angeles, California). Immunoglobulins were quantitated in radial immunodiffusion plates (Hyland Laboratories). In some experiments serum proteins were evaluated by the double-gel diffusion method (12).

Results. A total of 102 human sera were treated either with kaolin or M-H to compare the effectiveness of the two methods to remove the nonspecific inhibitors of rubella-virus hemaggutination (Table I). Fifty-seven of these sera were tested for the amount of inhibitors present in human serum prior to any treatment. None of the sera was devoid of inhibitors and the titers ranged from 128 to >8192. Following a single absorption with kaolin the HI titers were reduced often to <8. Residual HI was presumed to be due to antibody specific for rubella virus. To test this the neutralizing antibody titer of some of these sera was determined. All but 2 sera that had HI activity after kaolin treatment also had neutralizing antibody. The 2 exceptions were sera that had HI titers of 8 and 16 without neutralizing antibody being detected in the original, untreated samples. On the other hand, 27 sera had no HI activity after kaolin absorption but 4 of these contained detectable amounts of neutralizing antibody. There was

TABLE I. Comparison of HI Titer of Kaolin Treated Sera with HI Titers of Original and M-H Treated Samples and with Neutralizing Antibody Titers.

No. of sera tested[a]	HI titers before and after treatment			Neutralizing antibody titers
	Before	Kaolin	M-H	
1	>4096	>1024	>1024	—
4	512–4096 (2901)[b]	1024	64–1024 (784)	—
17	1024–8192 (2757)	512	256–1024 (602)	64–128 (96)
17	1024–>8192 (3499)	256	32–512 (401)	16–128 (62)
14	1024–>8192 (4535)	128	16–512 (257)	16–64 (39)
8	1024–8192 (4864)	64	16–256 (155)	16 (16)
8	1024–4096 (2560)	32	16–512 (179)	8–32 (22)
4	1024 (1024)	16	64–128 (80)	4–32 (11)
0	—	8	—	—
23	128–2048 (5802)	<8	<8	<4
1	—	16	<8	<4
1	—	8	<8	<4
4	—	<8	8–64	8–16
Total 102	Av 2777			

[a] All sera were tested for HI titers after kaolin and M-H treatment, 57 sera were tested for HI titers before treatment and 53 sera were tested for neutralizing antibodies.

[b] Average titer of sera tested in this group.

a 95% correlation between the HI and neutralizing antibody titers when the sera for the HI test were pretreated with kaolin.

Aliquots of the same sera were treated with M-H as described in "Material and Methods" and the HI titers were determined. Forty-seven sera had the same HI titers after either treatment. The titers of another 46 sera were 2- to 8-fold higher after M-H treatment than after kaolin treatment. However, the HI titers of 9 sera after M-H were lower. Excluding 2-fold differences for these comparisons 84 sera had the same titers, 15 had titers $\geq$ 4-fold higher, and 4 had significantly lower HI titers in the M-H treated samples.

Two questions could be asked about these results. First, was the higher HI titer frequently obtained after M-H treatment due to specific antibody, or was it due to residual nonspecific inhibitors? The answer to this question could best be obtained from those sera that contained HI activity after M-H, but not after kaolin, treatment. Four such sera (Table I) contained neutralizing antibody thus the discrepancy was due to false negative titers of the kaolin-treated samples. The second question was whether M-H treatment removed HI antibodies since 6 M-H treated serum samples had significantly lower HI titers than after kaolin treatment. Two of these had HI titers of 8 and 16 after kaolin, and <8 after M-H treatment. Neither serum possessed demonstrable neutralizing antibody. Therefore the HI titers of M-H treated samples more closely reflected the occurrence of neutralizing antibody than did the HI titers of kaolin-absorbed sera. In addition, 10 sera tested for HI and neutralizing antibodies and found to be devoid of HI antibody after M-H treatment were also free of neutralizing antibody. The nonspecific inhibitors of HA did not interfere with virus infectivity in this test system. As shown in Table II neither kaolin nor M-H affected the

TABLE II. Comparison of Kaolin and M-H Treatment on HI and Neutralizing Antibody Titers with Original Serum Titers.

	HI test			Neutralization test		
	Orig.	Kaolin	M-H	Orig.	Kaolin	M-H
L8	2048	<8	8	8	8	8
L20	1024	<8	8	8	8	8
L21	256	<8	8	16	16	32

TABLE III. Effect of M-H Treatment on Serum Immunoglobulins.

	IgM[a]		IgG[a]	
Serum	Before	After	Before	After
HD	110	106	1230	1250
AT	120	112	1400	1385
EG	105	108	975	968
MA	75	72	650	652
MB	80	80	635	608
MH	40	37	350	338
JG	47	39	385	375
MJ	80	76	660	640
MAd	90	88	820	800

[a] mg/100 ml.

neutralizing titers of 3 sera tested. Thus false-negative HI tests were not observed in M-H treated sera.

Mann *et al.* (5) showed recently that M-H treatment quantitatively removed the β-lipoprotein from human serum. This fiinding was verified in this laboratory. Specific immunoglobulins were determined in 9 sera before and, in the respective supernatants, after M-H treatment. The treatment had no measurable effect on the levels of the IgG or IgM remaining in the supernatant (Table III). The redissolved precipitates were tested by the double diffusion method for β-lipoprotein. Precipitin bands developed between the wells containing the M-H precipitates, but not the M-H supernatants, and the β-lipoprotein antiserum.

Discussion. None of the human sera tested in this study was initially devoid of the nonspecific inhibitors of rubella-virus hemagglutination. The titers ranged from 128 to >8192 and were always greater than the specific HI antibody titers. Thus dilution alone would not allow recognition of even high titers of antibody. This demonstrated the importance of removing these nonspecific factors from human serum.

The kaolin treatment currently used as recommended by Stewart *et al.* (1) can remove the nonspecific inhibitors from serum. It has been criticized that kaolin is not always an effective absorbent and that it may remove specific antibody (2,13). In the present study there was a 95% correlation in the results of neutralization and HI tests when serum samples were treated with kaolin for the HI test. Similar results have been found by others (2,3), although a 100% correlation has also been reported (13). In order to obtain a greater degree of confidence in the HI test as an indicator of immunity to rubella a method is needed that would remove the nonspecific inhibitors from serum without altering immunoglobulins.

Little information is available concerning the nature of the nonspecific inhibitors of rubella-virus hemagglutination. In preliminary studies performed in this laboratory it was found that the nonspecific inhibitors in fetal calf serum (1) and in human serum could be removed by organic solvent extraction. This suggested that the inhibitors were lipid, or lipoprotein in nature. Organic solvent treatment of sera was not considered suitable because immunoglobulins could be denatured in the process. However, heparin in the presence of certain divalent ions was found to remove β-lipoprotein from human serum which confirmed the findings of Mann *et al.* (5) without altering the immunoglobulins of serum.

The effect of M-H treatment on nonspecific inhibitors and on specific antibodies of rubella virus HA was examined. Aliquots of 102 sera were treated either with kaolin or with M-H and HI titers were determined in parallel. Ninety-eight percent of the M-H samples had the same, excluding 2-fold differences, or higher HI titers than did the samples treated with kaolin. In 4 M-H treated samples the HI titers were ≧ 4-fold lower. Insufficient amounts of 2 of these sera prevented further examination. The other 2 sera had HI titers of 8 and 16 after kaolin absorption and <8 after M-H treatment and neither serum had neutralizing antibody. In a separate experiment involving only 3 sera it was found that the nonspecific inhibitors of HA did not affect the neutralizing titer of serum. Excluding those sera for which neutralizing antibody data were lacking, these results indicated that false-positive, or false-negative HI titers did not occur in M-H treated sera.

Technically the M-H method of preparing sera for the HI test is simple and rapid, and the reagents needed are stable and readily

available. It should be pointed out that M-H does not remove the natural agglutinins of 1-day-old chicken erythrocytes from human serum under the conditions employed here. It was necessary to absorb the M-H treated sera with erythrocytes to remove these agglutinins. For this reason it was essential to include serum-erytrocyte controls with each serum tested.

Summary. The nonspecific inhibitor(s) of rubella-virus hemagglutination were removed from human serum by the rapid, simple, and specific procedure of adding manganous chloride and heparin to the serum. This procedure was shown to precipitate preferentially β-lipoprotein without altering the immunoglobulin level in serum. The rubella-virus HI antibody titers after M-H treatment were equal to, or greater than, that after kaolin absorption and correlated in a high degree to neutralizing antibody titers.

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The Hyperglycemic and Antidiuretic Activity of a Phthalimidine Analogue: 1-Oxo-3-(4′-chlorophenyl)-3-hydroxyisoindoline (C3/76)* (33072)

JOHN K. WALES,[1] PETER BRADLEY,[2] JOSEPH VIKTORA AND FREDERICK W. WOLFF
(Introduced by C. Davison)
Research Foundation of the Washington Hospital Center and Department of Medicine, Division of Clinical Pharmacology, The George Washington University, Washington, D. C. 20010

Diazoxide, a nondiuretic benzothiadiazine, has already been shown by several investigators to cause hyperglycemia by inhibition of insulin secretion in some species (1, 2). Continuous search for drugs with fewer side effects and a prolonged action is stimulating interest in the structure–activity relationships of diazoxide as well as in the chemical similarities of diazoxide to the diuretic benzothiadiazines. Chlorothiazide, a member of the latter group of drugs, is widely used in clinical practice and has been reported to cause hyperglycemia. Chlorothalidone, which has a different chemical structure than the benzothiadiazines, has also been reported to cause hyperglycemia and importantly causes a similar but prolonged diuresis (3).

We prepared an analogue of chlorthalidone in which the sulfamyl group had been removed. The compound is 1-Oxo-3-(4′-chlorophenyl)-3-hydroxyisoindoline (Fig.

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[1] Present address: Department of Medicine, Post-Graduate Medical School, Hammersmith Hospital, DuCane Road, London W. 12, England.

[2] Present address: 69 Manor Road, Barnet, Herts, England.