by dissolving the sample in hot water and recrystallizing from pyridine. Its melting point was approximately 195°C. It appeared to be identical with known cellobiosazone that was examined at the same time.

This formation of a β-glucoside from an α-glucose derivative confirms the observation of Fitting and Doudoroff (*3*) that the synthesis of a disaccharide by this type of a phosphorylase involves a Walden inversion of the glucose-1-phosphate (*7*).

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Mechanism of Agglutination of Macaca rhesus Erythrocytes by Human Hepatitis Serum

In an attempt to find a useful laboratory method for the assay of the human hepatitis virus, we have investigated the recently described (*1*) phenomenon of *Macaca rhesus* erythrocyte agglutination. Although this reaction was considered to be the result of a nonspecific antibody combination, we examined the possibility that it might be a direct effect of a virus.

Hepatitis sera were obtained from local hospitals and from the Communicable Disease Center, U.S. Public Health Service, Chamblee, Ga. Many of the diagnoses were proved by autopsy or biopsy, and all had extensive laboratory support. For comparison, serum was obtained from other medical cases, both with and without jaundice, and from normal blood-bank donors of the various major blood groups and Rh types.

Blood from a small group of normal rhesus monkeys was drawn into 4 percent sodium citrate, and the cells were washed with large volumes of 0.9 percent normal saline. Hemagglutination reactions were carried out in twofold serum dilutions in saline; 0.2-ml volumes, measured with calibrated pipettes, were used. Equal volumes (0.2 ml) of the washed 1 percent rhesus cells were added, and the shaken tubes were refrigerated at 4°C for 2 hours. Agglutination was determined by the settling patterns and from the cohesion of the cells when the tubes were gently tilted.

The distribution of hemagglutination titers (reciprocal of the highest dilutions showing clear agglutination) among controls and medical patients, including those with clinical jaundice of nonviral origin, was homogeneous and ranged from less than 4 to 64. Neither blood type nor jaundice caused any detectable variation from the normal range. Saline-typing sera of the major groups and Rh types (anti-C, anti-D and anti-E) failed to produce any agglutination of the monkey cells.

Among the sera from hepatitis cases, both infectious hepatitis (IH) and serum hepatitis (SH) sera gave titers ranging into the thousands, usually 1024 or 2048, and occasionally higher. When serial samples were taken, the SH sera retained the higher titers both initially and throughout the recovery period. Some continued to show high readings even when no further clinical or biochemical evidence of hepatitis could be detected. On the other hand, the titer of the IH cases dropped off at approximately the same rate as the other criteria of the disease.

Of the 51 sera from clinically active cases of viral hepatitis tested, all showed titers exceeding 128. More than half of these were 1024 or over. In 22 sera from convalescents showing no clinical symptoms or biochemical abnormalities, about half were in the normal range, while the others ranged between 128 and 2048. There appeared to be no correlation between any of the clinical or biochemical tests and the level of hemagglutination.

Among convalescent SH cases, two with no detectable agglutination titers were mixed (in equal volumes) with both active IH and SH material and incubated at 37°C. The agglutination titers of all SH sera tested were rapidly and uniformly reduced (Fig. 1); but no decrease of effect was seen with any IH serum. However, none of the sera from convalescent IH cases tested had any effect on either IH or SH, under the above conditions. Commercial gamma globulin, which has a reproducible titer of 32, also failed to reverse the effect of either IH or SH material. Of ten normal sera tested, none had ability to reduce the hemagglutinating activity of any serum studied.

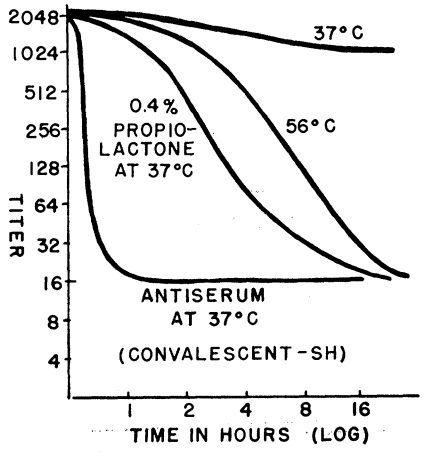

Fig. 1. Effect of various treatments on the agglutination of *Macaca rhesus* erythrocytes by the serum of an acute case of serum hepatitis (SH).

High-titer IH and SH sera were incubated at 37°C and at 56°C for up to 30 hours. Periodically, samples were tested for hemagglutination. Typical results are seen in Fig. 1. The lower temperature had only a slight effect, while the higher temperature effectively reduced the titer. The lower the initial titer, the more rapid was the reduction by heat.

A number of active serum samples were incubated under various conditions with β-propiolactone, a compound known to be strongly viricidal (*2*). The rate of disappearance of the hemagglutination titer depended on the concentration of the chemical and the temperature of incubation. A typical result is shown in Fig. 1. Higher concentrations at elevated temperatures were more rapidly effective. Refrigeration of serum containing 0.4 percent β-propiolactone caused only a slight loss of activity during the test period.

All the mixtures that were studied contained penicillin and streptomycin, and all were tested for sterility at the end of the experiment. No contaminations were observed, nor did the antibiotics alone interfere with the hemagglutination reaction.

In some cases treatment with heat, β-propiolactone, or serum from convalescents reduced the hemagglutination titers of active sera to less than four (the minimum test level). Usually, however, as shown in Fig. 1, there was an irreducible minimum below which the titer would not fall. In control runs, in which the three test treatments were compared in nonhepatitis sera that showed appreciable titers (32 to 64), no diminution of hemagglutination could be achieved. These methods also failed to lower the agglutinating ability of antisera experimentally prepared against the same monkey erythrocytes.

Our data could be explained by the presence of two independent mechanisms for the agglutination of rhesus erythrocytes by human sera. Clear low levels of effect are found in most human serum, regardless of the source and these seem to be of a nonspecific nature. But superimposed upon these levels there exists a

greater effect that may be reversed by at least three mechanisms known to be capable of reducing virus activity. These same mechanisms are without effect when tested against nonhepatitis serum, normal gamma globulin, or antiserum prepared with the test erythrocytes.

The sera from convalescents which were effective in reducing the effect of the hepatitis sera showed clear specificity, although we have not yet found a serum capable of reversing the IH serum effect.

It is also clear that at least the higher agglutination titers could not be accounted for by any of the major Rh blood types. This agrees with an early observation of Wiener (*3*) that human Rh negative sera capable of reacting with Rh human erythrocytes were ineffective against rhesus red cells.

It is possible, therefore, to account for the high hemagglutination titers as a virus effect and to remain consistent with the few known characteristics of the hepatitis viruses (*4*).

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Sulfa Compounds in Reversible Inhibition of Sperm Metabolism by Carbon Dioxide

A recent report from our laboratory has presented evidence of reversible inhibition at 37°C of motility and glycolysis of bovine spermatozoa by anaerobiosis and relatively high tensions of CO_2 (*1*). The potential for maintenance of fertility of spermatozoa inhibited by CO_2 during storage for 1 week under the rigors of normal room temperature has been demonstrated (*2*).

The Illini variable-temperature diluent (IVT) used in the studies of fertility contained 20.0 g of sodium citrate dihydrate, 2.1 g of sodium bicarbonate, 0.4 g of potassium chloride, 3.0 g of sulfanilamide, 1 million units of penicillin G, and 1.0 g of dihydrostreptomycin sulfate per liter. The sulfanilamide, penicillin, and streptomycin were first added to this diluent primarily as antibacterial agents to prevent the rapid bacterial growth that otherwise occurs during storage of bull semen at room temperature (*3*). In addition to its bacteriostatic effect in diluted semen, sulfanilamide has been known for more than 10 years to inhibit respiration of spermatozoa at 37°C and to inhibit aerobic utilization of carbohydrate by bull semen that is stored in yolk-citrate at 5°C (*4*). Sulfanilamide improved the fertility of semen used routinely for artificial insemination of cattle (*5*). Thus, sulfanilamide has been an ingredient of most diluents used for storage of semen for several years. However, only recently has it been found that sulfanilamide also has a supplementary effect on the inhibition of anaerobic glycolysis brought about by relatively high levels of CO_2 in N_2. Neither penicillin nor streptomycin exerts such a marked inhibitory effect.

Table 1. Carbon dioxide evolved per 10^8 spermatozoa from bicarbonate during 4 hours at 37°C in Illini variable temperature diluent (IVT) supplemented with 0.02*M* sulfanilamide or 0.001*M* Diamox in N_2 plus CO_2 and CO_2 only.

Carbon dioxide (%)	IVT only (μl)	IVT and sulfa (μl)	IVT and Diamox (μl)
5	97	50	49
50	30	13	13
100	14	8	6

The function of sulfanilamide as a competitive inhibitor of *p*-aminobenzoic acid is recognized (*6*), as is its inhibition of phosphatases (*7*) and of carbonic anhydrase of animal origin (*7*). Zinc (*8*), a known component of carbonic anhydrase, and phosphatases have been found in bull semen.

It is the purpose of this report to present the evidence that sulfanilamide supplements the inhibition of metabolism by CO_2. Another compound, Diamox, or 2-acetylamino-1,3,4-thiadiazole-sulfonamide (*9*), which is considered as a specific inhibitor of carbonic anhydrase (*7*), also inhibits glycolysis but does so at a lower concentration.

For these studies, 0.2 ml of freshly collected bull semen, containing from 200 million to approximately 450 million sperm cells, was added, in Warburg flasks, after temperature equilibration to 37°C, to 1.0 ml of the Illini variable-temperature diluent containing none of the antibacterial agents. The diluent served as the control when the flasks were gassed for approximately 10 minutes with CO_2 or with N_2 containing 5 or 50 percent CO_2. The diluent in other flasks contained sulfanilamide (0.02*M* or Diamox (0.001*M*). The results reported in Table 1 are the mean cumulative evolution of CO_2 from bicarbonate by 10^8 spermatozoa for three semen samples during a 4-hour incubation at 37°C.

The presence of sulfanilamide and Diamox depressed the glycolysis of the spermatozoa to a level much below that in the Illini variable-temperature diluent alone at all levels of CO_2. Most of the glycolytic activity in the presence of these two additives at the 100-percent-CO_2 level occurred during the first 15 minutes of incubation. When either sulfanilamide or Diamox was used, the inhibition of glycolysis was as effective in 50 percent N_2 and 50 percent CO_2 as that occurring in the diluent under an atmosphere of pure CO_2. The recovery of spermatozoan motility after the incubation and after aeration upon opening the flasks was optimum in the diluent alone and in that with sulfanilamide added but was depressed slightly by Diamox, the mean values being 55, 55, and 44 percent, respectively. Diamox levels higher and lower than the 0.001*M* level were not as effective in controlling glycolysis and did not improve the recovery of spermatozoan motility upon aeration after incubation.

The effect of sulfanilamide on the glycolytic activity of CO_2-inhibited spermatozoa has been repeatedly confirmed. In comparisons with 15 additional semen samples, the number of microliters of CO_2 produced per 10^8 sperm cells in 4 hours at 37°C in the absence and presence of sulfanilamide were 95 and 56, 24 and 12, and 11 and 8 under 5, 50, and 100 percent CO_2, respectively.

These supplementary inhibitory effects of sulfanilamide and Diamox were not due to differences in *p*H. The mean final *p*H's of the flask contents were 6.7, 7.0, and 6.7 in 5-percent CO_2 for the Illini variable-temperature diluent alone, with sulfa added, and with Diamox added, respectively. With 50-percent CO_2 the final values were 6.8, 6.6, and 6.7; with 100-percent CO_2, they were 6.7, 6.7, and 6.5, respectively.

The above results confirm the earlier report of CO_2 inhibition of glycolytic activity of spermatozoa and show that at least two sulfa compounds increase the inhibitory effect of CO_2. The mechanism of sulfa inhibition as well as CO_2 inhibition of spermatozoan glycolysis remains to be identified (*10*).

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