concert with the other known prime regulators of calcium homeostasis, parathyroid hormone and calcitonin, and (v) is effective in alleviating some of the pathological conditions of vitamin D–resistant rickets, osteomalacia, sarcoidosis, and uremia, all diseases which have been postulated to be due to abnormalities in CC metabolism.

It is possible that 1,25-dihydroxycholecalciferol should be reclassified as a steroid hormone. Certainly its secretion by the kidney followed by its selective accumulation in the target intestinal mucosa where it exerts its characteristic physiological effect on calcium metabolism satisfies the minimal definitions of a hormone. Norman has previously commented on the analogies in the mode of action of CC to that of several other steroid hormones, particularly aldosterone, testosterone, and estrogen (*16*).

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10. Only some 4 to 6 pmole or 2 to 3 ng of metabolite 4B are present in the intestine obtained from a 110-g chick (*1*).
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14. The lipid was applied in diethyl ether to a 30-g (1 by 80 cm) column of silicic acid. The column was batch eluted sequentially with 200 ml of 100 percent diethyl ether (25-hydroxycholecalciferol), 300 ml of 50 percent diethyl ether in dichloroethane (vol/vol) (metabolite 4B), and 200 ml of 100 percent methanol (other polar metabolites). The metabolite 4B fractions were concentrated under nitrogen and applied to a 1 by 80 cm column of Sephadex LH-20 eluted with chloroform-petroleum ether (65 : 35). Metabolite 4B emerged between 275 and 325 ml. These fractions were concentrated under nitrogen and applied to a 20-g (1 by 80 cm) Celite liquid-liquid partition column which was eluted with a mobile phase of 20 percent (vol/vol) 1,2-dichloroethane and 80 percent petroleum ether saturated with a stationary phase of methanol-water (90 : 10 by volume) as described previously (*2*). The metabolite 4B was eluted after 700 to 800 ml, concentrated under nitrogen, and subjected to silica gel thin-layer chromatography. The silica gel had been prewashed with 100 percent acetone, followed by 100 percent diethyl ether, and dried, and the plates were preeluted with 100 percent ethyl acetate to remove trace lipids. After application of the metabolite 4B sample to the plate, it was developed with ethyl acetate : petroleum ether (93 : 7). In this system cholecalciferol, 25-hydroxycholecalciferol, and metabolite 4B have R_F's of 0.89, 0.76, and 0.45, respectively. The metabolite 4B was eluted from the gel with redistilled 100 percent methanol, and concentrated under nitrogen prior to mass spectrometric analysis.
15. The kidney homogenate incubations employing 25-hydroxycholecalciferol (25-OH-CC) were found to be one-step metabolic transformations. That is, only one major compound more polar than 25-OH-CC was found in an extensive chromatographic analysis of the incubation media (*11*).
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Fertilization of Rabbit Ova in vitro by Sperm with Adsorbed Sendai Virus

Abstract. *Fertilization occurs when rabbit ova are cultured in vitro with epididymal sperm to which Sendai virus is adsorbed. These sperm do not require capacitation in vivo in order to fertilize. Evidence for fertilization is penetration of sperm, the appearance of two polar bodies and pronuclei, and cleavage through the eight-blastomere stage. The viruses attach almost exclusively to the sperm acrosome, with resultant head-to-head agglutination of the sperm.*

Chang (*1*) in 1959 submitted incontestable evidence for fertilization in vitro; live young were born after transplantation of cleaved rabbit ova to foster mothers. Others have confirmed and extended the technique (*2, 3*). Common to these studies was the use of sperm which have undergone a physiological change (capacitation) within the female tract. Several workers have attempted, with partial success, to capacitate rabbit sperm in vitro (*4*), but all successful studies of fertilization of rabbit ova in vitro have used sperm capacitated beforehand in vivo. We report here the fertilization of rabbit ova in vitro with sperm that have not been capacitated in vivo. Originally we mixed Sendai virus with washed ejaculated sperm for a short incubation with ova. Some of these ova underwent cleavage when transplanted 3 to 5 hours later to recipient does. In this work we used epididymal sperm, because more agglutinate with the same amount of virus; improved equipment permitted longer incubation of ova. Criteria for fertilization are penetration of sperm, production of the second polar body, formation of pronuclei, and cleavage (*5*). The experiments are reproducible with approximately 50 percent of ova cleaving (Table 1).

Dutch-belted and New Zealand does were bred to vasectomized males or injected intravenously with 125 I.U. of chorionic gonadotrophin (National Biochemical Company). Their freshly ovulated ova were recovered by flushing of the oviducts 12 hours later with culture medium TC-199 kept at 37°C (flushing was carried out at room temperature). Gametes were incubated at 37°C in a 35 by 10 mm petri dish with 1.5 to 2.0 ml of TC-199 (*p*H 7.6) and 100 units of penicillin and 100 μg

Table 1. In vitro fertilization of rabbit ova by epididymal sperm with adsorbed Sendai virus. From these and other experiments an additional 159 (sperm with virus), 22 (sperm control), and 26 (virus control) ova were observed before cleavage for evidence of fertilization 3 to 12 hours after culture. In the table, those ova which had cleaved had reached the two- to eight-cell stage.

Experiment	Sperm with virus		Sperm control	
	Ova cultured (No.)	Ova cleaved (No.)	Ova cultured (No.)	Ova cleaved (No.)
1	8	3		
2	31	8	12	0
3	15	4	12	0
4	22	16		
5	15	5		
6	38	28		
7	15	9	25	0
8	25	9		
9			36	0
10	16	9		

of streptomycin per milliliter; the incubator had an atmosphere of 5 percent CO_2 in air at saturated humidity. Sperm from the cauda epididymidis were suspended in TC-199 to a density of 1×10^7 sperm in 0.05 ml. Sendai virus, prepared by passage of stock virus in allantoic fluid of 9- to 10-day-old chicken embryos, was mixed [0.2 ml of 1×10^4 hemagglutinating units (HAU) per milliliter] with the 0.05 ml of sperm and kept at room temperature for 15 minutes before incubation with ova. Controls included ova with virus only plus ova and sperm without virus. Ova were transferred 3 hours later to TC-199 plus 5 percent heated rabbit serum; some were observed for evidence of fertilization at that time and thereafter through 24 hours. Denuded ova, obtained by vigorous shaking of the ova (*6*), were viewed unstained with a phase contrast microscope and then fixed and stained (*7*) for further microscopic observation. The virus was stored at −18°C or at −56°C in Hanks basic salt solution until used. At −18°C the HAU remained the same after 6 months of storage, but the ability of virus to agglutinate sperm or elute from agglutinated chicken red blood cells decreased with storage time. Only virus samples which agglutinate sperm and red blood cells and elute from the latter were used.

Motile sperm upon being mixed with virus immediately agglutinate head-to-head (Fig. 1). The agglutination reflects an interaction between sperm and virus which is thought necessary for altering sperm physiology. Electron micrographs (*8*) show that viruses attach almost exclusively to the acrosome, the head region which releases enzymes upon capacitation. Compared to ejaculated sperm, a higher percentage of epididymal sperm agglutinate when mixed with virus. Even though washed, ejaculated sperm contain more debris as competing binding sites. Gamete viability and fertility is apparently not altered by the presence of the live virus. Ova 7 to 8.5 hours after incubation are in second meiotic division or forming the second polar body, or the second polar body is present with pronuclei forming to include syngamy. Sperm are present in the perivitelline space (unstained ova reveal none to less than five sperm which are mostly nonmotile); however, the fertilizing sperm tail has not been identified with certainty. Control sperm

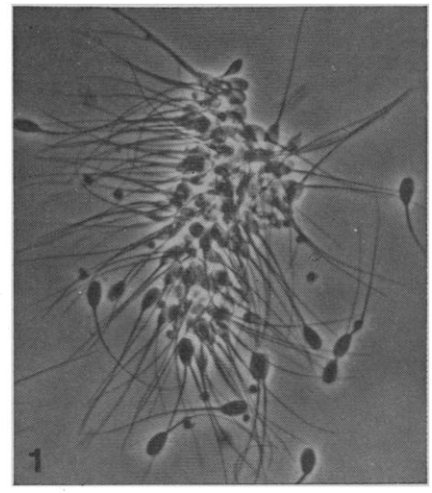

Fig. 1. Head-to-head agglutination of live epididymal rabbit sperm after being mixed with Sendai virus. Phase contrast, × 630.

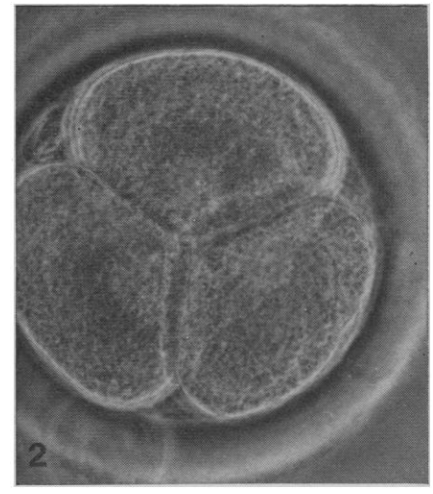

Fig. 2. Denuded four-cell stage rabbit ovum viewed 25 hours after culture in vitro. Phase contrast, × 630.

do not penetrate the ova. Forty-two ova placed with virus do not show evidence of artificial activation; the second polar body does not form nor do the ova cleave. Cleavage of fertilized ova up to eight blastomeres within 25 to 28 hours of incubation provides the best evidence of normalcy. Fifty-two percent of 91 ova are in the two-cell stage (Table 1) when viewed, and the remainder are in the four- to eight-cell stage (Fig. 2). The experimental reliability of obtaining cleaved ova compares favorably with earlier work in which sperm were capacitated in vivo (*2*). Ova do not cleave when placed with control epididymal sperm (Table 1).

Sendai virus fuses membranes of many somatic cells (*9*), and we thought, therefore, that it might alter sperm membranes. If virus-induced membrane vesiculation occurs, then sperm enzymes necessary for penetration of the ovum are liberated. We do not know the mechanism of virus-activated sperm fertility in vitro. A reasonable guess is that sperm undergo a form of capacitation and acrosome reaction. The acrosome reaction which occurs near the ovum (*10*) is a fusion of plasma and outer acrosome membranes. Normally capacitation must take place first before the acrosome reaction can occur; the virus may act to induce a combination capacitation-acrosome reaction. Electrostatic forces, and particularly the negatively charged molecule sialic acid, are responsible for attachment of myxovirus particles to cells (*11*). We do know that Sendai virus adsorbs to sperm and perhaps drastically changes the sperm surface by a viral enzyme's splitting off a negatively charged molecule. These virus particles, seen almost exclusively on the anterior sperm head, are probably lost before sperm penetration of the ovum (*10*). Rabbit sperm will, however, incorporate simian virus 40 genome, which is subsequently carried into the ovum at fertilization (*12*). Others have established that viruses infect fertilized ova by passing through the zona pellucida (*13*).

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