ences in intake between 21- and 22-hour deprivation, between 22- and 25-hour deprivation, and between 25- and 30-hour deprivation (all differences at $p < .01$). It is interesting, too, to note that, although a significant increase in intake occurs between 0- and 4-hour deprivation in the schedule condition, there is a nonsignificant *drop* in intake between 4- and 21-hour deprivation.

Scheduling of drinking on a once-a-day cycle seems to have the effect of reducing differences in intake between some relatively short period of deprivation (in this case, 4 hours) and longer periods up to the deprivation interval associated with scheduled drinking, and then of producing a sharp rise in intake at deprivation intervals longer than the one normally associated with scheduled drinking.

A second study was run in order to make a closer examination of intake at the lower end of the deprivation range. Ten albino Wistars were studied, five with free access to water at all times other than test intervals and five under 23-hour scheduled deprivation. Tests of water intake were run at 0, 1, 2½, 4, 5, 6, and 12 hours of water deprivation. In this study, the results of which are shown in Fig. 2, intake over the first 10 minutes of the test hour was analyzed because it had been shown that intake during the shorter period adequately represented intake over the entire hour.

It should be noted in Fig. 2 that, as the first study suggested, the rise of intake with deprivation appears to be sharper in the schedule group than in the free access group. For example, intake for the schedule group was significantly different ($p < .01$) between the lower deprivation intervals (1- and 2½-hour) and the higher intervals (6- and 12-hour). The rise of intake in the free access group, although regular, did not reach conventional levels of statistical significance.

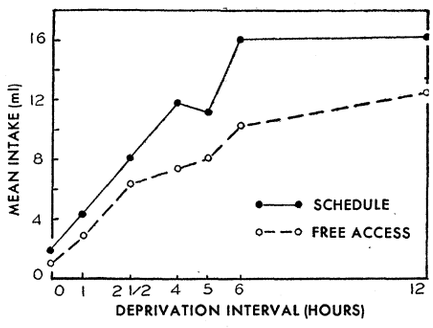

Fig. 2. Amount of water drunk during a test period of 10 minutes following water deprivation of varying intervals for animals with free access to water and for animals with access to water limited to 1 hour a day.

These studies confirm the observation of earlier workers (*3*) that a sharp rise in consummatory behavior takes place between 0 and 4 to 6 hours of deprivation, but they also suggest that part of this effect may be ascribable to the influence of caretaking schedules. Animals which have been "trained" to drink every 24 hours will show distortions of intake when compared with animals tested from a base line of free access to water. These distortions suggest caution in the use of hours of deprivation as a simple index of "thirst" and lend support to Miller's conclusions (*4*) about the complexity of drive measures in general.

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2 February 1960

Effects of Polyvalent Cobalt Salts on Human Cells in Tissue Culture

Abstract. Trivalent and hexavalent cobalt complexes have been tested to determine their ability to produce morphological changes in two different human cell cultures. Aortic intimal cells which produce mucopolysaccharides in vitro prove to be more susceptible than the nonmucopolysaccharide producing HeLa strain. This effect seems to be related to the reaction of polyanions such as ribonucleic acid, deoxyribonucleic acid, and chondroitin sulfate, with the complex cobalt salts.

The use of polyvalent cobalt salts for the precipitation of chondroitin sulfate and deoxyribonucleate (*1*, *2*) prompted us to investigate the effects of these salts on freshly grown cells capable of synthesizing sulfated mucopolysaccharides. Trivalent hexamminecobaltic chloride, $Co(HN_3)_6Cl_3$, and hexavalent hexol nitrate, $[Co(OH)_6(Coen_2)_3](NO_3)_6$, prepared by Maxwell Schubert (*3*), were examined for purity by x-ray powder diffraction patterns (*4*). The trivalent salt in dilute aqueous solutions forms precipitates with sulfated mucopolysaccharides and nucleic acids (RNA as well as DNA), and their respective protein complexes. At physiological *p*H and salt concentrations, only DNA is precipitated (*2*). The hexavalent salt will also precipitate hyaluronate and react with polyanions at physiological *p*H and ionic strength. That the cobalt salt may also form soluble complexes with polyanions is indicated by the dissolution of such precipitates by excess anion, and a specific increment at 235 mμ produced in a solution of hexamminecobaltic chloride by the presence of chondroitin sulfate (*3*).

To test whether these salts could precipitate the mucopolysaccharides or polysaccharide-protein complexes of aortic cells specifically, or both, several human aortas were extracted. Water soluble aortic mucopolysaccharide-protein complex was obtained by Schubert's method (*5*, *6*). This material was metachromatic, had a clear absorption peak at 280 mμ in a Beckman DU spectrophotometer, and contained 9.8 percent hexosamine and 8.2 percent nitrogen. It was obtained in a yield of 1 to 2 percent of dried, defatted aorta and sedimented as two peaks in the analytical ultracentrifuge. In aqueous solution the material gave precipitates with both polyvalent cobalt salts directly proportional to the amount of polysaccharide-protein complex added.

Due to the relative specificity of the reaction, we proposed to study whether these salts would (i) enter cells growing in tissue culture and (ii) manifest effects either in cytoplasmatic sites of RNA and mucopolysaccharide localization or in nuclear DNA itself. Two human cell types were used: (i) intimal cells from adult human aorta organ cultures (*7*) which produce mucopolysaccharides in short-term studies (*8*), and (ii) a commercial stock of strain HeLa (*9*) (Microbiological Associates, Inc.). Both cell types were maintained in 10 percent human serum and 90 percent Eagle's basal medium for periods of up to 120 hours.

The cobalt salts were dissolved in triple distilled water with a magnetic stirrer, sterilized by filtration through a UF fritted disc (Corning) in a Morton filter apparatus, and diluted to concentrations of 1 to 150 μg/ml in sterile Hanks' solution. After the drug was added, flying-coverslip type cultures were incubated at 36.5°C and observed periodically. The cultures were fixed in 10 percent Ringer's formalin and stained with May-Grünwald-Giemsa by W. Jacobson's method (Strangeways Laboratory), Alcian blue 8 GS-Ehrlich's hemalum, and periodic acid-Schiff.

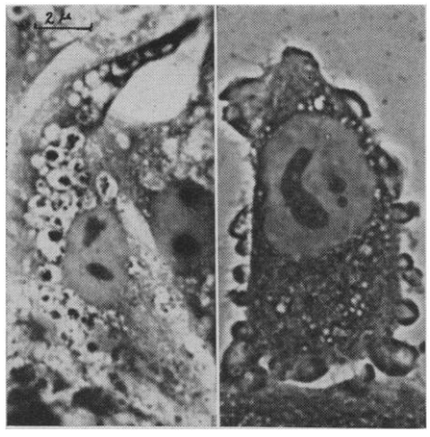

Fig. 1 (left). Cytoplasmatic vacuoles in human aortic intimal cells produced by trivalent hexamminecobaltic chloride. (Right) Intranucleolar granules in human aortic intimal cells produced by hexavalent hexol nitrate.

Half of the cultures were incubated for 12 hours after the drug was added, rinsed twice in Hanks' solution, and maintained in normal culture medium for the rest of the incubation time. As is shown in Table 1, different morphological nuclear and cytoplasmatic changes were observed in the aortic intimal cells and in HeLa cells at various concentrations of the salts. Trivalent hexamminecobaltic chloride at concentrations of 5 μg/ml produced in aortic intimal cells large cytoplasmatic vacuoles containing metachromatic granules after 12 hours of incubation, but no nuclear changes (Fig. 1, left). Such effects were not observed in the HeLa cells under similar conditions. Both cell types show growth inhibition and typical cytotoxic effects at concentrations of 100 μg/ml.

Table 1. Effect of various salts on human cell cultures.

Concn. (μg/ml)	Human aortic intimal cells		HeLa cells	
	Cytoplasmatic	Nuclear	Cytoplasmatic	Nuclear
	$Co(NH_3)_6Cl_3$			
1	±	−	−	−
5	+	−	−	−
25	++	−	−	−
50	+++	−	−	−
75	+++	−	−	−
100	+++	±	±	±
	$Co(OH)_6(Coen_2)(NO_3)_6$			
1	−	±	−	−
5	−	+	−	−
25	±	++	−	−
50	++	+++	−	±
75	+++	+++	±	+
100	+++	+++	+	++
	$CoCl_2$			
250	±	−	±	−
	$Ni_2(NH_3)_6Br_2$			
200	±	−	±	−

Hexavalent hexol nitrate (5 μg/ml) produced intranucleolar granules but no cytoplasmatic changes in human aortic intimal cells. At concentrations of 50 μg/ml these granules were very abundant and were accompanied by formation of large bubbles in the cell membrane but no vacuolization (Fig. 1, right). The nucleolar changes persisted for up to 120 hours of cultivation if these cells were exposed to the salt for 12 hours. The salt did not inhibit their mitotic index or prolong their generation time. In HeLa cells this salt produced nuclear picnosis, growth inhibition, and cytolysis, at concentrations of 100 μg/ml.

Control tests carried out with cobaltous chloride at similar concentrations showed growth inhibition with 250 μg/ml in both cell types without any of the morphological changes described above. Nickelous ammonium bromide produced similar results at concentrations of 200 μg/ml.

The effects of trivalent hexamminecobaltic chloride in aortic intimal cell cytoplasm can be interpreted as the induction of dilated vacuoles of endoplasmic reticulum with metachromatic granules. They are of interest because such findings have not been observed in HeLa cells, which in our experience have never shown production of mucopolysaccharides in tissue culture. No mitotic changes like those described for cobalt nitrate and other sulfhydryl reagents (*10*) were observed in any of these two cell lines. The effects of the hexavalent salt in the nucleolus and its persistence after removal of the salt from the culture medium are particularly pertinent considering the very low concentrations of this salt required to react in vitro. The action of these cobalt salts seems to be different from those observed by Levy *et al.* (*11*) with cobaltous sulfate in bacteria (*Proteus vulgaris*), which was able to arrest protein synthesis without halting RNA production.

Our results suggest that at very low concentrations trivalent cobaltous salts act upon cytoplasmatic RNA and sulfated mucopolysaccharide while the hexavalent salt reacts more specifically with nuclear RNA. These results support the hypothesis that complex cations capable of reacting with polyanions in solution exert morphological effects upon living cells at sites where polyanions are present, their degree and site of action depending on the cell type used and on the ability of the cells to produce mucopolysaccharides in vitro (*12*).

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Hypothesis Concerning the Role of Follicular Contractions in Ovulation

Abstract. Autotransplants of ovarian tissue in the anterior chamber of the eye may be studied in the lightly anesthetized rabbit. Individual follicles in such implants, consisting of two to five follicles, have been observed to contract after subcutaneous injection of urine of pregnant women, after application of rat pituitary homogenate to the cornea, and 9 to 10 hours after cervical stimulation.

Although the presence of smooth muscle in the theca externa of the ovarian follicle of the rabbit was first reported by Thomson (*1*), neither he nor Guttmacher and Guttmacher (working with the sow) (*2*) succeeded in demonstrating functional activity in this muscle. The Guttmachers in 1921 attempted to induce the smooth muscle of the sow's ovarian theca externa to contract by means of electrical excitation and application of acid, alkali, and barium chloride solutions. These stimuli, where successful, were unphysiological, and although these workers suggested that the follicular muscle coat might be involved in the mechanism of ovulation, they failed to substantiate their hypothesis.

The observations reported here define a physiological role for the smooth muscle present in the theca externa. Such a role has been largely discounted because of two sets of experimental observations. Friedman (*3*) demonstrated that ovulation could occur in ovarian implants in the rectus muscle, and Hinsey and Markee (*4*) found that ovulation occurred in the denervated ovary.

Autotransplants of small numbers of ovarian follicles in the anterior chamber of the eye may be studied in the