B_{12} in sea water was tested with water collected in July 1957 from two stations in Long Island Sound and frozen within 5 hours after collection. For the assay, the sea-water samples were enriched with glucose, glutamate, lysine, thiamine, and agar at the level of the basal medium. Five- and 2.5-ml portions of each sample were diluted with full-strength basal medium (without bicarbonate) to a final volume of 10 ml. Undiluted samples were inhibitory to *Thraustochytrium globosum* in the presence or absence of added vitamin B_{12}. Consistent results and satisfactory recovery of added vitamin B_{12} (5 μμg/ml) were obtained with one sample at both dilutions; with the other, only at the greater dilution. Both samples assayed 16 to 20 mμg of vitamin B_{12} per liter—a level somewhat higher than that found for unfiltered ocean water at other locations and at various seasons by Cowey (*5*) and by Droop (*6*) (0.2 to 4.0 mμg/lit and 5 to 10 mμg/lit, respectively).

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4 November 1957

Propagation of Infectious Canine Hepatitis Virus in Porcine Kidney Tissue Culture

Infectious canine hepatitis virus has generally been considered to be a host-specific agent (*1*). Numerous attempts have been made, in this laboratory and elsewhere, to adapt this virus to embryonated eggs, mice, rabbits, and other animals, with essentially negative results. The successful propagation of infectious canine hepatitis virus in dog-kidney tissue cultures with the production of a typical cytopathogenic effect has been reported (*2, 3*). By continuous passage in dog-kidney tissue culture, the virus was modified so that it did not produce disease when it was inoculated into susceptible dogs (*3*). Previous attempts in this laboratory to adapt virulent infectious canine hepatitis virus to pig-kidney explants were unsuccessful. This report shows that the virus can be successfully propagated in pig kidney epithelial cells after a series of passages in dog-kidney tissue cultures.

Tissue cultures were prepared from the kidney cortex of pigs of between 2 and 12 weeks of age by a trypsin digest method in which 0.33 percent trypsin was used. Two nutrient media were employed: (i) 0.5 percent solution of lactalbumin hydrolyzate in Earle's balanced salt solution, fortified with 10 percent inactivated horse or bovine serum (ELS medium); (ii) medium No. 199 (Parker) without serum. Each medium contained penicillin and streptomycin. Initial attempts to adapt infectious canine hepatitis virus to trypsinized-pig-kidney tissue cultures were made with virulent virus. From portions of liver from two dogs killed during the acute phase of infection, 20-percent suspensions in physiological saline were made, and the supernatants of these were inoculated into culture tubes containing ELS medium. Additional cultures were inoculated, in the same manner, with tissue culture fluid from the 14th dog-kidney tissue culture passage of infectious canine hepatitis virus. The cultures, incubated at 35°C, were observed for 7 days, after which time the fluids from each group of tubes were harvested and passed into additional tubes. Subpassages of each group of cultures were made three times, at 7-day intervals. At that time, the tissue culture fluids were inoculated into trypsinized-dog-kidney roller tubes, to test for the presence of virus. No virus could be detected.

Further efforts at adaptation were made with modified virus which had undergone 134 passages in dog-kidney cultures. Several pig-kidney tissue cultures in tubes containing ELS medium were inoculated in a manner similar to that described above, and subpassages were prepared. Commencing with the fifth tissue culture passage, a portion of the cells exhibited a cytopathogenic effect that resembled, to some degree, the changes previously observed in dog-kidney tissue cultures. This effect continued to occur, in an erratic manner, through the tenth passage. Beginning with the tenth passage, the nutrient medium was changed to No. 199. Thereafter, a complete cytologic change developed in the cells 3 to 6 days after inoculation and occurred regularly through 28 additional passages. The titer of the tenth pig-kidney passage was $10^{5.0}$, as was demonstrated by titrations in dog-kidney tissue culture. Titrations at intervals through the 38th passage ranged from $10^{4.5}$ to $10^{5.5}$. The affected cells became swollen, rounded-up, and highly refractile. Epithelial sheets broke up, and the cells formed small, grapelike clusters.

Serum neutralization tests to identify the cytopathogenic agent in pig-kidney tissue were performed at various passage levels. Infected tissue culture fluid prepared in tenfold dilutions was mixed with an equal amount of known infective-canine-hepatitis-positive canine antiserum. These mixtures were incubated for 2 hours in a 37°C water bath and inoculated, in 0.2-ml amounts, into each of several pig- and dog-kidney cultures. Up to 100,000 tissue culture infectious doses of virus were neutralized both in pig and dog cultures by the positive serum, while the usual cytopathogenic effect occurred in control cultures that contained the normal serum.

Tests were made in dogs to provide additional evidence that the cytopathogenic agent propagated in pig-kidney tissue culture was infectious canine hepatitis virus. Fourteen infectious-canine-hepatitis-susceptible dogs were used. Eight were inoculated subcutaneously with 19th-passage, and six with 34th-passage, material. Daily temperature recordings and observations for other signs of illness were made for 3 weeks, following which the animals were challenged with known virulent virus. No signs of illness were observed in the dogs inoculated with pig-adapted virus, and none of the dogs responded to challenge. Preinoculation serums were found to be negative (by a serum-neutralization test employing known infectious canine hepatitis virus that had been grown in dog-kidney culture); all dogs had developed neutralizing antibody titers of from 100 to 3200 three weeks later. The development of specific antibodies in the dogs further demonstrates that the cytopathogenic agent is infectious canine hepatitis virus. The lack of illness in the inoculated dogs indicates that the virus is of modified virulence and could be used for development of a vaccine.

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5 August 1957

Action of Hydrocortisone on Respiration and Aerobic Glycolysis of Cultured Cells

In previous reports, inhibition of growth of fibroblasts by a single high dose of hydrocortisone was demonstrated (*1*). Cells derived from a single fibroblast, which had been isolated from a culture of normal subcutaneous tissue of the mouse, Earle's strain L, have been

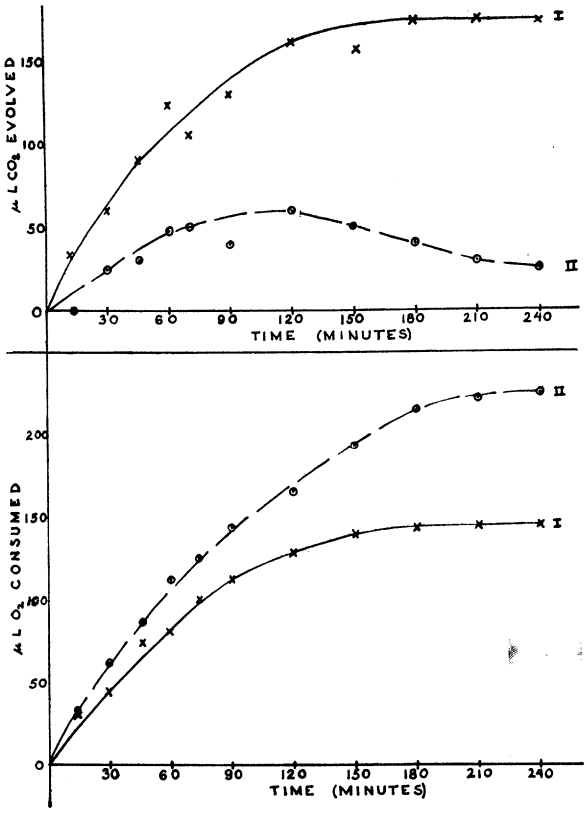

Fig. 1. (Top) Aerobic glycolysis: curve I, 150 μg of hydrocortisone per milliliter; curve II, control. (Bottom) Respiration: curve I, 150 μg of hydrocortisone per milliliter; curve II, control.

found to be highly responsive to the growth-inhibiting action of hydrocortisone and were chosen for the present experiments (*2*). Growth measurements were carried out by the method of enumeration of cell nuclei (*3*). In 11 experiments the average growth inhibition caused by a single dose of hydrocortisone in a concentration of 125 μg/ml was found to be 40 percent. In order to obtain more information concerning the mechanism of the direct action of hydrocortisone on cells, its action on respiration and aerobic glycolysis of cultured cells has been investigated.

Two series of experiments were carried out. In the first series, the cells from eight T-60 flasks, each containing about 15×10^6 cells growing in a medium consisting of embryo extract, horse serum, and Earle's balanced salt solution in proportions of 20:40:40, were scraped from the glass, pooled, and centrifuged; the residue was suspended in 25 ml of fresh medium containing phenol red as a *p*H indicator (*p*H approximately 7.4). Three milliliters of the cell suspension was put into each of the eight Warburg flasks, and the remaining 1 ml was used for enumeration of cell nuclei. To four of the Warburg flasks, 0.50 ml of Earle's salt solution containing hydrocortisone in a concentration of 125 μg/ml was added, and to four control flasks, 0.50 ml of Earle's solution, without hydrocortisone. Since the hydrocortisone stock solution contained 50 percent ethanol, ethanol in equal final dilution was added to the control vessels. Control and experimental flasks in most cases were in duplicate: two control flasks containing 0.20 ml of 15 percent KOH in the central well, for measurement of oxygen uptake, and two control flasks containing 0.20 ml of H_2O in the central well, for measurement of aerobic glycolysis; two flasks for measurement of oxygen uptake in the presence of hydrocortisone, and two for measurement of aerobic glycolysis in the presence of hydrocortisone. The reading obtained from the flasks with H_2O in the central well is the resultant of both the consumption of O_2 (as shown on the flasks with alkali) and the liberation of CO_2. The aerobic glycolysis is thus obtained by taking the difference between the readings on the flasks with and without alkali. The "direct method" (*4*) was employed because, for comparison of values obtained in the presence and in the absence of hydrocortisone, the effect, if any, of CO_2 on the rate of respiration was assumed to be insignificant.

In the second group of experiments, to four of eight T-60 flasks, each containing equal numbers of cells, hydrocortisone in a concentration of about 1.50 μg/ml was added at the time of explanation. After 72 hours the same concentration of hydrocortisone was added to each of the four flasks (without removal of the medium), plus 2.50 ml of new medium. After another 24 hours (96 hours from the beginning of the experiment), hydrocortisone in the same final concentration was again added to the cell suspensions of the four Warburg flasks in new medium, before they were put in the manometric bath. Each Warburg flask contained in its main compartment equal numbers of cells suspended in 3 ml of new medium, to which 0.50 ml of Earle's salt solution containing hydrocortisone was added. Four control vessels were treated in the same way, but without hydrocortisone. In four experiments, in which only respiration was measured, cholesterol, cortisone acetate, and the mineralocorticoid deoxycorticosterone in the same concentration, were also used as controls.

In the first group of experiments, a single dose of hydrocortisone, when added to the cell suspension in Warburg flasks, caused inhibition of respiration which, in eight out of nine experiments, was coupled with significant increase of aerobic glycolysis. Hydrocortisone in the same concentration, when added in a single dose to cultured cells, causes growth inhibition averaging 40 percent for 125 μg/ml, as determined by enumeration of cell nuclei. In this group of experiments, 40 μg/ml appeared to be below the minimal concentration of hydrocortisone necessary for obtaining reproducible inhibition of respiration by a single dose added immediately before the experiment (Fig. 1). Accordingly, concentrations of about 40 μg/ml in a single dose showed no significant growth inhibition of cultured cells.

In the second group of experiments, hydrocortisone added to the cultures at 96 hours, at 24 hours, and immediately before the manometric experiment, caused significant stimulation of respiration and of aerobic glycolysis.

It appears that the inhibition of growth and the inhibition of respiration by the same high concentration of hydrocortisone are connected with one another.

In the first group of experiments, the release of glycolysis may be due to the inhibition of respiration, in accordance with the "Pasteur effect," while in the second group of experiments, in which low concentrations of hydrocortisone were used, the stimulation of both respiration and aerobic glycolysis undoubtedly represents a specific action of the glucocorticoid hydrocortisone. The mineralocorticoid deoxycorticosterone did not have the same effect as hydrocortisone, and cholesterol was without effect.

While hormonal effects on anaerobic glycolysis have been demonstrated recently in certain tumor tissues (*5*), the present studies emphasize the possibility of demonstrating reproducible hormonal effects in aerobic glycolytic systems. The latter may reflect more truly the conditions prevailing in the organism.

The relationship between the high aerobic glycolysis of hydrocortisone-treated cells and the decrease of "resistance" to tumor grafting in hydro-

cortisone-treated animals can at the present time be only a matter of conjecture.

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11 October 1957

Effects of Stimulation of Brain Stem on Tachistoscopic Perception

This report concerns part of a study (*1*) conducted on rhesus monkeys to investigate the effects of electrical stimulation of different sites in the brain on tachistoscopic perception.

For this investigation the animals were first trained to discriminate between stereometric objects presented in pairs, by placing food reward under one of the objects of each pair. After this preliminary training without restriction of exposure duration, each animal was subjected to several series of trials at different exposures; the number of correct responses was noted, and the reaction time was automatically measured. The testing apparatus was a modification of that used by Harlow, which for our purposes was transformed into a tachistoscope (Fig. 1). Its main feature was an argon-mercury bulb which was used to flash briefly a light of controllable duration and constant intensity upon the objects. The pairs of white objects (for example, a cone and a 12-sided pyramid of similar proportions) were placed in a dark field in front of the animal. A brief acoustic signal preceded by 2 seconds the illumination of the objects; this signal was in no case concomitant with the visual stimulus. Selection of the correct member of each pair was always followed by a food reward. The position of the correct member was changed randomly from trial to trial.

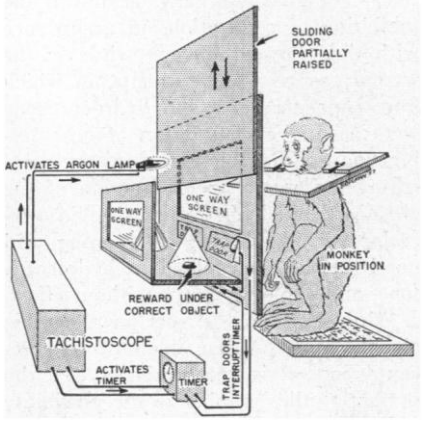

Fig. 1. Testing apparatus for visual discrimination. The monkey is shown in one of the plastic collars (schematically represented) by which the animals were permanently held to protect the electrode mount from their hands and to restrict them partially for performing the experimental task.

Electrodes were implanted in the animals' brains so that the effects of electrical stimulation could be studied. To date, six animals have been used in the investigation. The electrode placements were histologically verified. As a rule, currents were always used that did not cause any apparent effect on the normal behavior of the awake animal, regardless of the placement of the electrodes. A biphasic square wave current, of 300 cy/sec and intensities between 100 and 300 μa, was used as a norm. Each animal was used as its own control. In the experimental series, the electrical stimulation was applied during each trial period, starting 2 seconds in advance of the flash and persisting until the animal had made its choice.

Stimulation of the core of the brain stem at the level of the mesencephalon consistently increased the animals' efficiency at discrimination, as indicated by significantly higher percentages of correct responses and shorter reaction times as compared with the controls (Fig. 2). Stimulation through the same electrodes with intensities higher than the threshold for the elicitation of observable motor effects, such as generalized muscular jerks (startle reaction), eye movements, pupillary oscillations, vocalization, and so forth, had a deleterious effect on the performance of the animals, as indicated by the diminution of correct responses at all exposure durations and prolongation of reaction time. These effects were consistent and reproducible from animal to animal.

The same areas which upon mild stimulation facilitated tachistoscopic discrimination have previously been shown to evoke electroencephalographic and behavioral arousal in the sleeping or relaxed animal (*2, 3*). These areas are in the rostral part of the brain stem activating system, which is mostly composed of the reticular formation of the midbrain tegmentum. Evoked potentials of long latency to sensory stimuli are also picked up in these areas (*4*). This was verified in the present investigation, by using the same electrodes for recording.

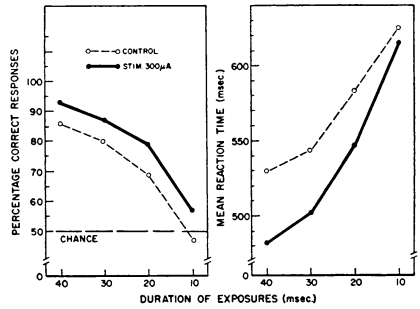

Fig. 2. Effects of stimulation of the brain stem on tachistoscopic discrimination. Each plotted point is based on 100 trials.

Both perceptual and motor processes involved in tachistoscopic discrimination appear to be facilitated by stimulation of the reticular activating system. It is difficult to determine whether the effects on reaction time are a direct consequence of the facilitatory effect on perception or independent of it. However, it seems likely that the reticular facilitation is primarily upon "central integrative time," rather than upon peripheral transmission time.

These findings give support to the hypothesis that the reticular activating system, whose primary role has been demonstrated to be that of central mediator for the achievement and maintenance of wakefulness by means of activation of the cortex, extends its function to the alert state as a further manifestation of the same physiological role, subserving basic attentive behavior. Its different degrees of excitation underlie the gradation of this function from deep sleep to extreme alertness. Excitation of this brain stem system induces general activity of the cortex (arousal), presumably facilitating its receptivity to the sensory impulses ascending over the classic sensory paths. The facilitation of tachistoscopic discrimination by electrical stimulation of the same system may be considered as an example of such an effect on visual perception.

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