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Cultivation of Measles Virus in Human Amnion Cells and in Developing Chick Embryo.* (23140)

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Enders and Peebles(1) presented evidence indicating that the virus of measles can be isolated in cultures of human and monkey renal epithelial cells from the blood and throat washings of patients in the early stage of the disease. Capable of continuous multiplication in such cultures, the agent produces a characteristic cytopathogenic effect which is specifically prevented by sera of man or monkey taken during the convalescent phase of measles. Moreover, an antigen appears in the fluid of infected tissue cultures that fixes complement specifically in the presence of convalescent phase measles sera(1,2,3). These findings have made it possible to investigate more conveniently and precisely the behavior of the virus in other systems. In this communication we shall describe the propagation of the virus and the cytopathic changes it induces in cultures of human amnion cells. In addition we shall record details of the procedures by which evidence of its multiplication in chick embryos was obtained. A summary account of these experiments has recently been published(2).

Methods and materials. Viruses. The "Edmonston" strain(1) of measles virus was most extensively employed. This agent was isolated in February, 1954, from the blood of a typical case of measles on the first day of the rash. From that time until January, 1955, it was subjected to a total of 24 passages in roller tube or trypsinized monolayer cultures of human postnatal renal cells maintained in bovine amniotic fluid medium. To a less extent certain other strains isolated in this laboratory were studied in respect to their capacity to multiply in human amnion cells and in chick embryos.

Tissue cultures. Suspensions of amnion cells were prepared according to the method of Zitcer and her coworkers(4). Approximately 300,000-400,000 cells in a volume of 1 ml of medium were allowed to settle on the wall of a tube 15 x 150 mm held at an angle of about 5° from the horizontal. During the period of cell outgrowth the medium consisted of the following constituents:

Normal horse serum (inactivated)	20 %
Bovine embryonic extract	5 %
Bovine amniotic fluid	37.5%
Hanks' balanced salt solution	37.5%
Streptomycin	100 mg/ml
Penicillin	100 units/ml
Mycostatin	100 "

In the most recent experiments the following medium, which gives equally satisfactory results, has been used to promote cell outgrowth:

	%
Normal horse serum (inactivated)	20
Hanks' balanced salt solution	70
L-Glutamine (2.9 mg/ml H_2O)	10

Antibiotics in quantities indicated in formula above.

When the monolayer was established and at the time the virus was introduced the medium used to promote cell outgrowth was replaced

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by a mixture of the following composition:

	%
Normal horse serum (inactivated)	5
Bovine embryonic extract	5
" amniotic fluid	45
Hanks' balanced salt solution	45

Antibiotics as given in first formula.

Suspensions of trypsinized human renal cells were prepared according to the procedure described by Younger(5) for monkey renal cells. The renal tissue was obtained at operations for reduction of hydrocephalus by ventriculo-ureteral shunt. The 5% normal horse serum-bovine amniotic fluid medium was employed throughout in the cultivation of these cells. *Embryonated eggs.* Technical details are included in the description of experiments on cultivation of the virus in chick embryos.

Results. Cultivation of measles virus in *human amnion cells.* Undiluted tissue culture fluid of the 24th human renal cell passage of the Edmonston strain was used as inoculum to initiate a series of 28 passages in monolayer cultures of human amnion cells. Volume of inoculum was 0.1 ml which contained $10^{2.8}$ TCD_{50} virus as determined in cultures of human renal cells.

Some difficulty was experienced in adapting the Edmonston virus to growth in the amnion cells. During earlier passages the cytopathic changes extended very slowly throughout the monolayer and, as compared with those seen in cultures of human renal cells, appeared after a longer interval, *i.e.* 3 to 4 days later. In attempts to adapt to human amnion cells 6 additional strains of measles virus that had been cultivated for a varying number of passages in human renal cells, inocula of 0.2-0.4 ml in 4 cases resulted in proliferation of virus when 0.1 ml proved ineffective. In the original amnion cell cultures of these strains, cytopathic changes were again slow to appear and in subsequent passages viral multiplication in certain instances did not occur.

Cytopathic changes in amnion cells. The changes induced by the Edmonston strain in human amnion cells are of 2 distinct types. The first consists in the syncytium-like or multinuclear giant cell formation which characteristically reflects the multiplication of the virus in cultures of human or monkey kidney cells(1,2). The syncytia are composed of a broad expanse of cytoplasm that includes numerous nuclei. Eosinophilic intranuclear inclusions are a prominent feature in this "lesion." Irregular masses of strongly eosinophilic amorphous material surrounded by a clear zone are often seen in the cytoplasm. Although doubtful at first respecting the significance of these cytoplasmic bodies, we have recently become convinced that they also are specific manifestations of cytopathogenicity, since we have never observed them in uninoculated cultures. The appearance of the syncytial change in an early amnion cell passage is shown in Fig. 1. The second type of change, (Fig. 2), consists in the assumption by an increasing number of epithelial cells of a spindle-like or, in certain instances, stellate configuration. Concomitantly refractility of the affected cells increases, rendering them conspicuous amid the more translucent normal cells. Margins of the elongated cytoplasmic processes distinguishing the affected cells are somewhat irregular or shaggy and occasionally exhibit a fine "beading." Usually these changes are first seen in a few cells lying adjacent to each other. Later much of the cell population becomes involved and the process slowly terminates in complete cellular disintegration. As passage of the virus in successive cultures of amnion cells was continued, the transformation of spindle cells

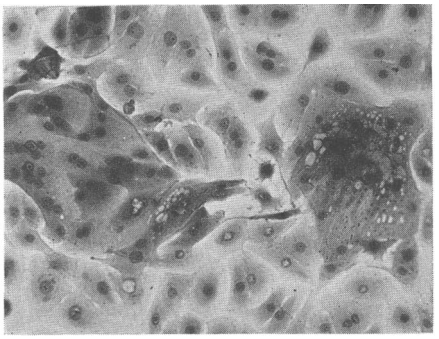

FIG. 1. Cytopathic changes in human amnion cells 16 days after inoculation of Edmonston measles virus of the 24th passage in human renal cells. Note syncytia or "multinuclear giant cells" and absence of spindle forms. Bouin's fixative. H & E stain. Mag. 107 ×.

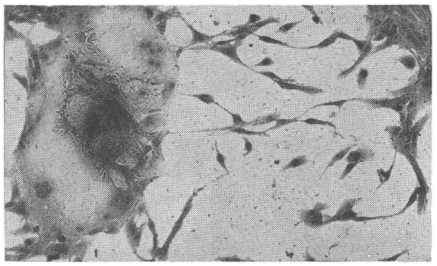

FIG. 2. Changes in human amnion cells 16 days after inoculation of Edmonston virus from 24th passage in these cells. Note spindle forms together with a syncytium in which irregular cytoplasmic inclusions are visible. Bouin's fixative. H & E stain. Mag. 85 ×.

tended to predominate over formation of syncytia. Indeed, in many cultures, particularly when small quantities of virus were inoculated, only spindle cells were seen. Examination of stained material revealed eosinophilic intranuclear inclusion bodies in certain of the spindle cells that closely resembled those occurring in the nuclei of the syncytia. The spindle cell change was first definitely associated with viral activity when in cultures of the 14th passage it was seen to involve a large proportion of the cell population. We now know from results obtained by reexamining the cytopathogenic effects of virus from certain of the earlier amnion cell passages that the Edmonston strain was capable of inducing this type of change at least by the fourth passage. Fluids from earlier passages were not available for test. This change, however, has not been observed in the original human amnion cell passages of 6 other strains of virus. Whether it will appear as serial passage of these agents is continued remains to be determined.

Spindle cell formation an attribute of measles virus. To show that the spindle cell transformation represents another expression of cytopathogenicity of the measles virus, neutralization and complement fixation tests were carried out with acute and convalescent phase human and monkey sera. The results are included in the first 2 rows of Table I. In these experiments tissue culture fluid of the 25th and 26th renal cell passages (designated "renal cell" virus) and the 28th amnion

TABLE I. Neutralization and CF Tests with Edmonston Virus Grown in Human Kidney and Amnion Cells and in Chick Embryos.

	Human sera*				Monkey sera†			
	Neut. test		CF test		Neut. test		CF test	
Source of virus	Acute	Conv.	Acute	Conv.	Acute	Conv.	Acute	Conv.
Human kidney P25 + P26‡	<8	177¶	<4	128**	<4	175	<4	32
Human amnion P28§	"	355	"	128	"	609	"	128
Egg P 1‖	—††	—	—	—	—	—	"	256
2	—	—	—	—	<4	2430	"	"
3	—	—	—	—	—	—	"	128
4	<8	1200	—	—	—	—	—	—
9	<16	609	—	—	<16	180	"	16

* Sera of patient from whom Edmonston strain was originally isolated. Acute phase specimen taken on 1st day of rash; convalescent 58 days later.

† Acute and convalescent sera from 3 monkeys were used: CF and neut. tests with virus from human kidney, human amnion and Egg P 9 were done with sera from Monkey 69 inoculated with 23rd human kidney passage Edmonston strain(6); sera from Monkey 78 inoculated with the same virus were used in neut. tests with Egg P 2; sera from Monkey 52 inoculated with the 1st passage in human kidney cells of the Edmonston strain(6) were used in CF tests with Egg P 1,2,3.

‡ Fluids from 25th and 26th passages in human kidney cells were respectively used in neut. and CF tests.

§ Virus from 28th passage in human amnion cells.

‖ Egg P 1 = first egg passage. Infected chick amniotic membrane suspensions were used in neutralization tests with egg-propagated virus. Pooled TC fluids from cultures of human amnion cells inoculated with virus from chick embryo passages were used as antigens in CF tests.

¶ Reciprocal of neutralizing titer of serum against 100 TCD_{50} calculated by formula of Reed and Muench.

** Reciprocal of highest dilution of serum fixing complement with 2 units of antigen. Tests carried out by technic of Fulton and Dumbell(7) as modified by Svedmyr and co-workers(8).

†† Not done.

TABLE II. Infectivity and Cytopathogenic Effect of Edmonston Virus in Cultures of Two Kinds of Human Cells.

Source of virus	Culture: Human kidney* CPE‡	Human kidney* Infect§	Human amnion† CPE	Human amnion† Infect
Hu am P28‖	Syncyt¶	3.5	Sp cells**	3.5
Egg P6††	"	3.8	"	3.8

* Trypsinized human kidney cells.
† " " amnion " .
‡ Cytopathogenic effect.
§ Infectivity titer: log TCD_{50}/0.1 ml.
‖ Fluid from 28th passage in human amnion cells.
¶ Syncytial formation.
** Predominantly spindle cell formation.
†† Pooled chick amniotic membrane suspension of 6th egg passage.

cell passage (designated "amnion cell" virus) were employed as antigens. The "renal cell" virus induced only formation of syncytia in cultures of human kidney cells whereas the effect of the "amnion cell" virus consisted predominantly or solely in development of spindle forms. Cultures of human renal cells were used in the neutralization tests with "renal cell" virus and cultures of human amnion cells in the tests with "amnion virus." One of the 2 pairs of sera was obtained from the patient yielding the Edmonston virus; the other from a cynomologus monkey that exhibited typical signs of measles following inoculation of fluid from the 23rd passage of this agent in human renal cells(6).

The data in Table I indicate that the acute phase sera in the lowest dilutions tested failed to prevent the cytopathic changes induced by either "renal cell" or "amnion cell" virus. In contrast the convalescent phase sera in high dilution proved inhibitory. Similarly, these sera brought about fixation of complement in comparable dilutions in the presence of both antigens while the active phase sera did not. Because these findings show that the virus capable of inducing the spindle cell change in amnion cells is antigenically similar or identical with the agent propagated only in human renal cells, it may be concluded that the measles agent itself is responsible for this newly recognized cytopathic effect.

Additional data supporting this conclusion were obtained in titrations of virus of the 28th amnion cell passage carried out simultaneously in cultures of human amnion and kidney cells. The results are summarized in the upper row of Table II. In both systems the endpoint of viral infectivity proved to be the same. In the renal cell cultures, however, only syncytia were observed, whereas only spindle cells were seen in the amnion cell series.

Cultivation of measles virus in the developing chick embryo. Nearly 2 decades ago several workers reported the successful propagation of measles virus in the developing chick embryo(9). Of these reports the most convincing were those of Rake and Shaffer(10, 11). Similar attempts by others, however, yielded inconclusive or negative results. This situation in retrospect is, perhaps, not surprising since at that time the only means of demonstrating the presence of the virus was by inoculation of monkeys and it has recently been demonstrated(6) that many normal monkeys possess naturally acquired immunity to the agent. Because of the conflicting results of these earlier workers, susceptibility of the chick embryo to infection with measles virus has remained uncertain, although in 2 recent communications, one from Japan(12) and the other from Russia(13), further evidence is presented suggesting that this agent may be propagated in this system.

When it was found that multiplication of measles virus could be accurately and conveniently demonstrated and measured in tissue culture, Enders and Peebles(1) attempted to repeat the experiments of Rake and Shaffer(11), using the tissue culture technic to determine presence or absence of the agent in various constituents of the chick embryo. Following the procedures described by Rake and Shaffer for establishment and maintenance of the virus in the developing hen's egg, no evidence of viral multiplication was obtained. Subsequently additional experiments with 5 strains of measles virus were carried out in this laboratory in which the same technics were employed. However, even in materials taken from the original egg passages, no virus was demonstrated.

Recognition of the capacity of the Edmonston strain to induce the spindle cell change

after many passages in cultures of 2 different kinds of human cells suggested that this newly observed cytopathic effect might be the result of a mutation which in turn might be accompanied by a change in the capacity of the virus to multiply in the chick embryo. Although results subsequently obtained have not supported this hypothesis, a series of egg passages was then initiated using as the original inoculum virus that had been cultivated during many passages in amnion cells.

Passage of virus in chick embryos. We have emphasized the fact that the technic employed in our unsuccessful attempts to cultivate the virus in this host was essentially that of Rake and Shaffer(11). Their usual procedure consisted in inoculation of the chorioallantois and the harvesting of materials for passage after an interval of 4 to 7 days. In the experiments to be described in which viral multiplication was finally demonstrated, the inoculum was introduced into the amniotic sac and the embryonic materials were collected as routine 9 days thereafter. These modifications were prompted by 2 considerations. The amniotic route was selected, since it seemed possible that the virus, now adapted to human amnion cells, might more readily proliferate in the analogous cells of the chick. The eggs were incubated for a longer period because in an experiment with virus from an earlier amnion cell passage the agent was recovered 9 days after inoculation of the embryos. Although at that time it was not demonstrated in subsequent egg passages, this finding suggested the possibility that slight multiplication may have occurred in the first passage after a period of incubation longer than that previously allowed.

In Table III are summarized details of procedure and the results obtained during 12 serial passages of the Edmonston virus in chick embryos. The original inoculum consisted of tissue culture fluid from the 28th human amnion cell passage. Chick amniotic or pooled amniotic and allantoic fluid was used as the inoculum in the first 6 sub-passages. Thereafter a pool of amniotic fluid and membrane suspension was employed, since by that time it had been determined that the amniotic membrane consistently contained the largest amount of virus. The em-

TABLE III. Serial Passages of Measles Virus: Edmonston Strain in Chick Embryos.

		Titer material harvested†				
		Amnion		Chorioallantois		Embryo
Passage	Inoculum*	Fluid	Tissue	Fluid	Tissue	
I	TCF hu am P28‡	+	NT§	—	NT	NT
II	Pooled fls	2.5‖	NT	2.5‖	NT	NT
III	*Idem*	.8	2.8¶	—	2.8¶	—
IV	Am fl	.5	3.5	.3	2.3	1.0
V	*Idem*	1.3	3.5	—	+	+
VI	"	.5	2.8	—	—	+
VII	"	—	1.5	—	—	—
VIII	Pool am fl and membr	—	3.5	—	—	—
IX	*Idem*	.8	3.5	+	+	+
X	"	1.8	2.8	+	+	+
XI	"	2.3	4.3	—	+	+
XII	"	2.3	4.5	NT	+	+

* Introduced into amniotic sac under direct vision. 5 embryos were inoculated with 0.2 ml of undiluted materials from previous passage except as noted.

† Infectivity titers expressed as log ID_{50}/0.1 ml. In certain instances where titrations were not done presence of virus in the material is indicated by "+"; failure to detect its presence by "—." Aliquots of 0.1 ml of undiluted materials or dilutions thereof were added to each of 3 cultures of human amnion cells. Based on an estimation of the wet volume, 10% suspensions of chick tissues were prepared in tissue culture medium for use in titrations or tests for presence of virus.

‡ Titer tissue culture fluid used as original inoculum = 4.0/0.1 ml; vol inoculated = 0.5 ml.

§ Not tested.

‖ Each of 2 sets of 5 embryos inoculated with pooled am and al fluids.

¶ Am and C-A membranes pooled and titrated. Collateral passage line initiated from 1 embryo dying on 8th day.

bryos were incubated for 6 days at about 38°C before the inoculum was introduced and at 36°C thereafter. The presence of virus in various embryonic constitutents was determined by adding the latter to cultures of human amnion cells. This procedure was necessary because no definite changes indicative of viral multiplication were observed within the contents of the egg itself. So far attempts have also failed to demonstrate the presence of viral complement fixing antigen and hemagglutinin in egg materials shown to contain the virus. Inspection of the titrational data (Table III) for the materials derived from successive passages leaves no doubt that viral multiplication occurred. Thus it may be conservatively estimated that the original inoculum by the 12th passage was diluted 10^{12} x taking the volume of fluid in the amniotic sac as 2 ml. This dilution factor exceeds by about 10^{7} x the number of infectious doses of virus introduced as inoculum in the first passage. Furthermore, beginning with the 4th passage the quantity of virus found in the amniotic membranes in most cases definitely exceeded that added as inoculum. With amniotic fluid of an embryo dying on the 8th day in the 3rd passage (see Table III), a collateral series of 8 egg passages was initiated. The amount of virus found in the various materials of each passage was in general comparable to those recorded in Table III. Similarly, during the course of 3 successive egg passages of another collateral line established with virus from amniotic membranes of the surviving embryos of the 3rd passage of the original line, evidence of multiplication was obtained in each passage.

Identification of virus. The agent propagated in the chick embryo was identified as measles virus in neutralization and complement fixation tests in which acute and convalescent phase human and monkey measles sera were employed. From the data recorded in the lower part of Table I it is apparent that titers of the convalescent sera in the presence of virus derived from various egg passages were of the same order of magnitude as those found for measles virus which had been maintained continuously in cultures of human renal and amnion cells. The identity of the

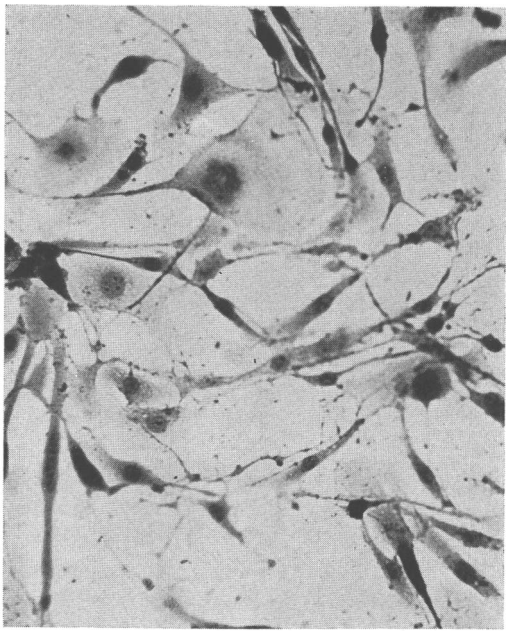

FIG. 3. Changes induced in human amnion cells by Edmonston virus of 3rd chick embryo passage 14 days after inoculation. Note predominance of spindle forms. A small giant cell is also visible, *Cf.* Fig. 2. Bouin's fixative. H & E stain. Mag. 230 ×.

agent cultivated in chick embryos was also supported by the fact that its cytopathogenic effect on human amnion cells, which consisted predominantly in spindle cell transformation, (Fig. 3), and on human kidney cells in which only syncytial changes were noted (Table II) was comparable to that of the virus used to initiate the series.

Discussion. The practical implications of the data presented in this paper will first be considered. Study of the measles virus during the last 3 years has been somewhat hampered by lack of large quantities of susceptible normal human cells free of contaminating agents. The finding reported here that the virus can be adapted to human amnion cells in which it induces characteristic cytopathic changes repairs this deficiency.

The results showing that at least one strain of virus can be propagated serially in the developing chick embryo may serve to remove any remaining doubts of the susceptibility of this host to the agent of measles. This demonstration is of importance, we believe, because of the many advantages the living chick

embryo or cells derived from it‡ would seem to offer in comparison with other media as a source of virus for the preparation of vaccines.

In considering the possibility of vaccination against measles, we may also emphasize the fact that the presence and amount of virus in chick embryonic materials can now be reliably and conveniently determined by means of tissue culture technics. Accordingly, attempts to immunize man against measles with egg-adapted virus can again be undertaken under more precise conditions of control than were available to workers in the past who have been concerned with this problem.

Several phenomena of more general biologic interest are presented by these observations. For example, recognition of a second and distinct cytopathogenic effect of the virus on human amnion cells has led us to inquire concerning the factors responsible for its appearance. Experiments are in progress to determine whether capacity to induce the spindle cell transformation is an original characteristic of the virus as it occurs in nature, or whether it emerged, possibly as a mutation, during the continued passage of the agent under the artificial conditions of the tissue cultures. Reissig, Black and Melnick(14) have recently shown with the Edmonston strain in cultures of human carcinoma cells that the cytopathic changes vary from the syncytial to the spindle form depending upon concentration of glutamine in the medium. At present, we believe that the phenomenon we have observed does not depend on this mechanism, since composition of the medium has remained constant, while the cytopathogenicity of the virus has varied.

The spindle cell transformation, as contrasted with the formation of syncytia, is also noteworthy, since it shows that wide departures from the "normal" or expected effect of a virus may occur within the same cell system. Indeed, in the case of measles virus these expressions of cytopathogenicity are so different that for a time we considered the possibility that the spindle cell change might have been induced by an unrelated agent introduced as a contaminant.

The factors which condition multiplication of the virus in the chick embryo have not yet been clearly defined. In the successful experiments, in contrast with those that failed, a longer period of incubation was allowed and another route of inoculation was employed. Furthermore, the virus employed as original inoculum had been subjected to many more passages in tissue cultures—an experience that may have increased its capacity to multiply in the chick embryo. Experiments have been undertaken to determine whether proliferation of the virus is dependent on any of these modifications.

Summary. 1. A strain of measles virus originally isolated in cultures of human renal cells has been propagated throughout 28 serial passages in cultures of human amnion cells. In the latter system it induces 2 types of cytopathic change: (1) formation of "syncytia" or "multinuclear giant cells" in which intranuclear and intracytoplasmic inclusions are prominent features; (2) only recently recognized, the assumption by individual epithelial cells of a characteristic fusiform or stellate configuration. In certain of these affected cells eosinophilic intranuclear inclusions are present that resemble those found in the nuclei of the syncytia. Eventually both types of change terminate in cellular necrosis and disintegration. In contrast to the dual response of amnion cells only the formation of syncytia has been observed in cultures of renal cells infected with the virus. 2. The Edmonston strain of measles virus from the 28th passage in human amnion cells was inoculated into chick embryos. In this host it has been maintained throughout 12 successive passages. Multiplication of the agent was demonstrated by addition of chick embryonic materials to cultures of human amnion cells. This procedure was necessary since no definite indication of viral activity has as yet been distinguished within the egg. 3. The virus present in the 9th chick embryo passage was identified as the measles agent in complement fixation

‡ Recently multiplication of the egg-adapted virus has been demonstrated throughout 6 serial passages in cultures of chick embryo cells. Details of these experiments will be published later.

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Decreased Oxygen Need as a Factor in Anemia of Hypophysectomized Animals.* (23141)

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Hypophysectomy induces an anemia in all animals studied, including the human. Various therapies have been shown to prevent this anemia in the rat, success being obtained with thyroxin and androgen(1), thyroxin, androgen, and a high protein diet(1,2), thyroxin and cortisone(3,4), adrenocorticotropic hormone(5), "erythropoietic hormone"(6,7), and cobalt(8,9). In spite of these results, the reason hypophysectomy induces an anemia is still under discussion. Some have claimed that loss of a pituitary erythropoietic factor explains it(6,7), while others have claimed that hypothyroidism and hypoadrenocorticalism, which inevitably follow hypophysectomy, are playing a role(10,11,3,4), although the pituitary seems to be involved in some manner which is in addition to its role in regulating thyroid and adrenal glands(11). Recent work whereby a combined therapy of growth hormone, thyroxin and cortisone returned all aspects of post-hypophysectomy anemia to near normal values‡ has indicated that growth hormone may be involved as well as thyroid and adrenal hormones.

These findings, however, do not explain the underlying mechanism of post-hypophysectomy anemia. The fact that oxygen consumption is decreased after hypophysectomy (12,13,14) and that anoxic anoxia is still considered to be the fundamental stimulant for erythropoiesis induced us to attempt to determine whether this anemia was correlated in any way with a decreased need for oxygen in hypophysectomized rats.

Methods. Unless otherwise indicated, adult female rats of the Wistar strain were used. All rats were fed Purina Chow *ad libitum* supplemented once a week with lettuce. Completeness of hypophysectomy was checked by observing the sella turcica with a binocular dissecting microscope at autopsy. Erythrocyte counts were done in duplicate using U.S. certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin deter-

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