OBSERVATIONS ON THE CULTIVATION OF VACCINE VIRUS IN LIFELESS MEDIA

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It is now an accepted fact that vaccine virus is capable of increasing *in vitro* in the presence of growing or surviving susceptible tissues. Most workers, however, have been unable to cultivate the active agent in lifeless media. Nevertheless, Eagles and McClean (1, 2) have reported that they successfully cultivated the virus in a "cell-free" medium. Moreover, Kendall (3) has suggested that his K medium is probably adequate for the *in vitro* growth of many viruses, including that of vaccinia. Inasmuch as the cultivation of vaccine virus in the absence of living cells would conclusively demonstrate that at least this virus is not only an autonomous entity but also one not possessing the characteristics of obligate parasitism, it seemed desirable to repeat Eagles and McClean's experiments and to test the importance of Kendall's suggestion. In this paper the results of our observations dealing with these matters are recorded.

EXPERIMENTAL

Eagles and McClean (1, 2) stated that they were able to obtain growth of vaccine virus in a medium consisting of a mixture of Tyrode's solution, rabbit serum, and an extract of rabbit kidney tissue. We have repeated their experiments according to the directions given in their reports. In view of the fact that vaccine virus multiplies in tissues other than those of the kidney, it seemed advisable to supplement Eagles and McClean's experiments by the use of extracts of rabbit testicles, rabbit spleens, and chick embryos in attempts to cultivate the active agent in lifeless media. Before undertaking the work with the tissue extracts it was essential for us to be able to cultivate the virus with regularity in the presence of the tissues themselves. The first part of the communication, therefore, deals with the results

of work on the cultivation of vaccine virus in media containing surviving cells derived from chick embryos, or from testicles, spleens, or kidneys of rabbits.

Virus.—The vaccine virus (4) used throughout the work is a dermal strain obtained from the New York City Board of Health. It can be propagated without difficulty by passages through rabbit testicles and is free from ordinary aerobic and anaerobic bacteria.

Tissues.—Testicular, renal, and splenic tissues were obtained from young rabbits (half-grown), washed in Tyrode's solution, placed in sterile watch-glasses contained in Petri dishes, minced with scissors, and then distributed in proper amounts to flasks by means of a pipette. Chick embryo tissue was obtained from eggs incubated 11 or 12 days.

Fluids.—Serum was collected from rabbits and diluted with 2 parts of Tyrode's solution. Tyrode's solution prepared according to the following formula was sterilized by filtration: NaCl, 8.0 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; NaH_2PO_4, 0.05 gm.; $NaHCO_3$, 1.0 gm.; glucose, 1.0 gm.; water, *q.s.* 1,000 cc.

Containers.—Carrel D flasks closed either by means of gauze plugs or rubber stoppers were used.

Preparation of Cultures.—Approximately 0.05 to 0.1 gm. of minced tissue to each 2 cc. of diluted serum was used. It is essential not to use too much tissue. The different tissues suspended in diluted serum (2 cc.) were distributed to Carrel D flasks. The media were then inoculated with vaccine virus and incubated at 37°C. for 3 or 4 days. New cultures were made by the direct transfer of the proper amounts of old cultures into flasks of fresh media. Cultures were usually prepared in duplicate or triplicate and were pooled before transfers or titrations were made.

Titration of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were prepared with Locke's solution. 0.25 cc. of each dilution was injected into the shaved skin of rabbits. The animals were observed daily for a week.

Minced Chick Embryo and Rabbit Testicular Tissue

In our laboratory it has never been difficult to cultivate (4, 5) vaccine virus in the presence of bits of minced chick embryo or rabbit testicular tissue. In fact, two sets of cultures, consisting of vaccine virus, minced chick embryo tissue, and Tyrode's solution are carried as a matter of routine. Consequently, further experimentation on these tissues seemed unnecessary prior to the work relating to the growth of the virus in their extracts.

Rabbit Kidney Tissue

Maitland and his coworkers (6–8) are able to cultivate vaccine virus in a medium consisting of bits of chicken or rabbit kidney suspended in a mixture of serum (1 part) and Tyrode's solution (2 parts). Eagles and McClean (1, 9) obtained similar results, but experienced difficulty in securing multiplication of the virus through a number of uninterrupted serial passages. Inasmuch as rabbit kidney had never been used extensively in our investigations, we decided to ascertain with what ease vaccine virus can be serially propagated in the medium employed by Maitland.

We made a number of attempts to cultivate vaccine virus in the presence of minced rabbit kidney and found that the active agent multiplied with sufficient irregularity to prevent uninterrupted serial passages. Then, in view of results obtained by Nigg and Landsteiner (10, 11) in the cultivation of rickettsiae, we decided to use different amounts of kidney tissue and to close some of the flasks with rubber stoppers instead of gauze plugs. One experiment of this nature, with the results obtained, is described and tabulated below.

Three lots of media, consisting of rabbit serum (1 part), Tyrode's solution (2 parts), and bits of rabbit kidney, were made in a similar manner with the exception that they contained different amounts of tissue and were designated as Lots A, B, and C. Lot A contained approximately 0.01 gm. of tissue for each 2 cc. of the mixture of serum and Tyrode's solution; Lot B, approximately 0.05 gm.; Lot C, approximately 0.35 gm. Each lot was inoculated with vaccine virus derived from a rabbit testicular emulsion. The titer of the virus in each lot was then determined by titration in the shaved skin of a rabbit. The different lots were divided into 2 cc. amounts which were distributed to Carrel D flasks. Rubber stoppers were used to close half the flasks of each lot, while gauze plugs were used for the other half. The cultures were incubated at 37°C. for 3 or 4 days. Similar cultures were then pooled and the titer of the virus was determined. In this manner serial passages of the virus were made and the results obtained are summarized in Table I.

The results of the experiment described above and summarized in Table I reveal that the virus multiplied in media containing small and medium amounts of kidney tissue but failed to grow in the mixtures of serum and Tyrode's solution in which large amounts of tissue were placed. The virus appeared to multiply equally well in the presence

of small and medium amounts of the tissue, and there was no marked difference in the results obtained that might be ascribed to the use of rubber stoppers instead of gauze plugs. The impression gained is, however, that one is able to cultivate the virus in series with less difficulty when rubber stoppers are employed. In any event, we have successfully cultivated vaccine virus through 18 uninterrupted serial passages in a medium consisting of a mixture of serum (1 part), Tyrode's solution (2 parts), and small amounts (0.01 to 0.02 gm. for each 2 cc. of fluid) of minced rabbit kidney placed in flasks closed by means of rubber stoppers.

TABLE I

Cultivation of Vaccine Virus in the Presence of Kidney Tissue

Time of titration	Lot A: titer of virus		Lot B: titer of virus		Lot C: titer of virus	
	Rubber stoppers	Gauze plugs	Rubber stoppers	Gauze plugs	Rubber stoppers	Gauze plugs
Before incubation	1,000	1,000	1,000	1,000	1,000	1,000
1st culture	>100,000	>100,000	>100,000	>100,000	100	100
2nd culture	100,000	100,000	100,000	100,000	±10	±10
3rd culture	>100,000	Contaminated	10	>100,000	−	−

Summary of results obtained in attempts to cultivate vaccine virus in the presence of different amounts of rabbit kidney tissue: Lots A, B, and C contained small, medium, and large amounts of tissue respectively. Some of the flasks were closed with gauze plugs, others with rubber stoppers. At the time the transfers were made, the virus underwent a tenfold dilution.

− indicates no active virus demonstrable.

Rabbit Splenic Tissue

It is not a surprising fact that we were able to cultivate vaccine virus in the presence of minced rabbit kidney because other workers have been successful. Nor were we surprised to find that too great an amount of minced tissue can be used in the media. When we undertook the cultivation of the virus in the presence of splenic tissue, however, we were unable to predict the results of the experiments, because Maitland and his coworkers (7, 8) had reported that they did not obtain multiplication of the active agent in the presence of minced

spleen suspended in a mixture of serum and Tyrode's solution. Nevertheless, we proceeded to repeat with splenic tissue the experiments performed with bits of minced kidney.

Two lots of media, consisting of rabbit serum (1 part), Tyrode's solution (2 parts), and splenic tissue from a young (half-grown) rabbit were made in a similar manner with the exception that they contained different amounts of the tissue, and were designated Lots A and B. Lot A contained approximately 0.005 to 0.01

TABLE II

Cultivation of Vaccine Virus in the Presence of Splenic Tissue

Time of titration	Lot A: titer of virus		Lot B: titer of virus	
	Rubber stoppers	Gauze plugs	Rubber stoppers	Gauze plugs
Before incubation	10,000	10,000	10,000	10,000
1st culture	10,000	>100,000	>100,000	>100,000
2nd culture	100,000	1,000,000	1,000	100,000
3rd culture	1,000,000	100,000	10	100,000
4th culture	100,000	100,000	–	10,000
5th culture	1,000,000	10,000	–	100,000
6th culture	100,000	–	–	–
7th culture	100,000	–	–	–
8th culture	100,000	–	0	0
9th culture	10,000	0	0	0
10th culture	100,000	0	0	0
11th culture	1,000,000	0	0	0

Summary of results obtained in attempts to cultivate vaccine virus in the presence of different amounts of splenic tissue of the rabbit: Lots A and B contained small and medium amounts of the tissue respectively. Some of the flasks were closed with gauze plugs, others with rubber stoppers. At the time the serial transfers were made, the virus underwent a tenfold dilution.

– indicates that no virus was present in cultures.

0 indicates that cultures were not prepared.

gm. of tissue for each 2 cc. of the mixture of serum and Tyrode's solution; Lot B, approximately 0.15 gm. Each lot was inoculated with vaccine virus cultivated in a medium containing minced chick embryo. The titer of the virus in each lot before the first period of incubation was determined by titration in the shaved skin of a rabbit. The two lots were divided into 2 cc. amounts and distributed to Carrel D flasks, half of which were closed with rubber stoppers while the others were closed with gauze plugs. The cultures were incubated 3 days at 37°C. and then the titer of the virus in each series was determined. Serial transfers into

fresh media were made at intervals of 3 or 4 days. The results of the experiment are shown in Table II.

From the results of the experiment summarized in Table II it is obvious that vaccine virus multiplied in the presence of rabbit splenic tissue suspended in a mixture of rabbit serum and Tyrode's solution. It also appears that the cultivation of the active agent was more regularly successful in media containing small amounts of the tissue placed in flasks closed with rubber stoppers. It seemed unnecessary to repeat the experiment because it was terminated while the virus was still multiplying satisfactorily.

Being assured that we were able to cultivate vaccine virus in the presence of minced chick embryo tissue or bits of tissue from the testicle, spleen, or kidney of a rabbit, we attempted to obtain multiplication of the active agent in media containing extracts of the tissues instead of the tissues themselves.

Kidney Extract

In the experiments dealing with the cultivation of vaccine virus in a "cell-free" medium we endeavored to follow the directions for the preparation of the "kidney extract" medium given by Eagles and McClean in their papers (1, 2) but encountered difficulty in determining how they proceeded. In one report (1) they stated that the "kidney extract" was used in place of the minced kidney tissue, but failed to give sufficient data, *e.g.* amounts of tissue and Tyrode's solution, to define how the extract was made. In another communication (2) they described the preparation of the kidney extract—3 to 4 cc. of Tyrode's solution added to a fresh rabbit kidney and centrifuged for 20 minutes—but failed to give details regarding the amounts of the different components in the medium, merely stating that the "kidney extract" was used in a culture medium consisting of "kidney extract, virus, serum and Tyrode's solution in amounts giving a final concentration of 1–100 of the virus." Inasmuch as we have seen no definite statements regarding the concentration of extract used, we have inferred that it was employed in approximately the same concentration as that designated by Maitland (6) for the minced tissues; *i.e.*, 0.66 cc. for each 20 cc. of the mixture of serum, Tyrode's solution, and virus emulsion. In order to avoid a serious error in the interpre-

tation of Eagles and McClean's directions for the preparation of the medium and to increase our chances of successfully cultivating the virus in a "cell-free" medium, we have, as will become evident from the work described below, used two concentrations of extracts in all experiments.

Kidney Extract.—While lightly anesthetized a half-grown rabbit was bled from the heart. The serum was collected for use in the media. Both kidneys—a kidney from a young rabbit weighs approximately 5 gm.—were then removed aseptically and thoroughly minced. To the minced tissue 6 to 10 cc. of Tyrode's solution were added and then the mixture was centrifuged at high speed for 20 minutes. The supernatant fluid was removed and again centrifuged for 20 minutes at high speed. This supernatant fluid was carefully removed and used in the preparation of the media. Two kinds of media were made, one of which was designated as dilute extract, the other as concentrated extract. The concentrated extract medium consisted of 3 cc. of serum and 7 cc. of undiluted kidney extract, while the dilute extract medium contained 3 cc. of serum, 1.0 cc. of kidney extract, and 6 cc. of Tyrode's solution. In a few experiments the dilute medium contained only 0.5 cc. of extract for each 10 cc. of mixture.

Cultures.—Concentrated and dilute extract media were inoculated—1 cc. of appropriate dilutions of virus emulsions for each 9 cc. of medium—with vaccine virus derived from rabbit testicular emulsions or cultivated in the presence of chick embryo tissue or rabbit kidney tissue. After inoculation the extracts were distributed in 2 cc. amounts to Carrel D flasks, some of which were closed with gauze plugs, others with rubber stoppers. A medium consisting of minced kidney tissue, serum, and Tyrode's solution was inoculated with virus and used as a control. Cultures were incubated at 37°C. for 3 or 4 days at which time serial transfers were made. The titer of the virus in the different media before the first cultures were incubated and in each set of cultures after incubation was established by animal experimentation. At the time transfers were made the virus underwent a tenfold, a twentyfold, or a hundredfold dilution.

A number of experiments, one of which is detailed below, were conducted in the manner just described.

Concentrated and dilute kidney extract media and a medium containing small amounts of minced kidney tissue were inoculated with vaccine virus that had been cultivated in the presence of kidney tissue. The dilute extract medium in this instance consisted of 1 cc. of kidney extract, 3 cc. of serum, and 6 cc. of Tyrode's solution. The titer of the virus in each lot of media was established by animal experimentation. The different media after inoculation were distributed in 2 cc. amounts to Carrel D flasks some of which were closed with gauze plugs, others with rubber stoppers. The cultures were incubated at 37°C. for 4 days and then

transfers were made to fresh media. Serial transfers were made in this manner every 3 or 4 days, the virus undergoing a twentyfold dilution at the time of each transfer. The results of the experiment are portrayed in Table III.

An examination of the results of the experiment summarized in Table III reveals that the vaccine virus did not multiply in the media containing concentrated or dilute kidney extract, while it did undergo an increase in titer in the medium containing bits of minced kidney. Eight other attempts were made to cultivate the virus in kidney extract media without success.

TABLE III

Cultivation of Vaccine Virus in Kidney Extract

Time of titration	Titer of virus in medium containing concentrated kidney extract		Titer of virus in medium containing dilute kidney extract		Titer of virus in medium containing bits of kidney tissue	
	Rubber stoppers	Gauze plugs	Rubber stoppers	Gauze plugs	Rubber stoppers	Gauze plugs
Before incubation.....	5,000	5,000	5,000	5,000	5,000	5,000
1st culture.....	Undiluted	—	—	—	1,000	100,000
2nd culture.....	—	—	—	—	>100,000	Undiluted
3rd culture.....	—	—	—	—	>100,000	—

Summary of results obtained in an attempt to cultivate vaccine virus in kidney extract media. Some of the flasks were closed with rubber stoppers, others with gauze plugs. The virus underwent a twentyfold dilution at the time of each serial transfer.

— indicates no active virus demonstrable.

Testicular, Splenic, and Chick Embryo Tissue Extracts

Efforts to cultivate vaccine virus in kidney extracts were supplemented by attempts to induce the active agent to multiply in extracts of spleens and testicles of half-grown rabbits or in extracts of chick embryos. Three experiments were performed with chick embryo extracts, one each with testicular and splenic extracts.

Splenic Extract.—Splenic extracts were made by the addition of 7 cc. of Tyrode's solution to the minced spleen (approximate weight 1.0 gm.) of a half-grown rabbit.

Testicular Extract.—Testicular extracts were obtained by the addition of 10 cc. of Tyrode's solution to the minced testicles (approximate weight 2 to 2.5 gm.) of a half-grown rabbit.

Chick Embryo Extract.—Chick embryo extracts were prepared by the addition of 10 cc. of Tyrode's solution to 2 minced chick embryos (approximate weight 2.5 gm.) 11 or 12 days old.

The extracts were rendered relatively cell-free by centrifugation twice at high speed for 20 minutes. Concentrated and dilute extract media were prepared, inoculated, and handled in a manner similar to that employed in the experiments

TABLE IV

Cultivation of Vaccine Virus in Testicular Extracts

Time of titration	Titer of virus in medium containing concentrated testicular extract	Titer of virus in medium containing dilute testicular extract	Titer of virus in medium containing bits of testicular tissue	Titer of virus in medium containing bits of kidney tissue
Before incubation	10,000	10,000	10,000	10,000
1st culture	—	—	10,000	10,000

Cultures incubated 3 days at 37°C. The flasks were closed with gauze plugs. The vaccine virus in the media containing dilute and concentrated testicular extracts was inactive after a 3 day period of incubation.

— indicates no virus was present in cultures.

TABLE V

Cultivation of Vaccine Virus in Splenic Extracts

Time of titration	Titer of virus in medium containing concentrated splenic extracts	Titer of virus in medium containing dilute splenic extracts	Titer of virus in medium containing a small amount of minced splenic tissue
Before incubation	10,000	10,000	10,000
1st culture	10	100	10,000
2nd culture	Undiluted	Undiluted	100,000

Summary of results obtained in attempts to cultivate vaccine virus in splenic extracts. Flasks were closed with rubber stoppers. Cultures were incubated at 37°C. and serial transfers were made at intervals of 3 days. The virus underwent a tenfold dilution at the time of each transfer.

with kidney extracts. In all experiments, however, the dilute extract media consisted of 3 cc. of rabbit serum, 1 cc. of tissue extract, and 6 cc. of Tyrode's solution. The results of the work are shown in Tables IV, V, and VI.

An analysis of the results of the experiments portrayed in Tables IV, V, and VI shows that the vaccine virus rapidly lost its activity in

media containing extracts of rabbit spleen, rabbit testicles, or chick embryo tissue. The titer of the active agent, however, either remained constant or increased in media containing bits of surviving tissues.

TABLE VI

Cultivation of Vaccine Virus in Embryo Tissue Extracts

Time of titration	Titer of virus in medium containing concentrated embryo extract	Titer of virus in medium containing dilute embryo extract	Titer of virus in medium containing a small amount of minced embryo tissue
Before incubation	100	100	100
1st culture	10	10	100,000
2nd culture	—	Undiluted	1,000,000
3rd culture	—	—	1,000,000

Summary of results obtained in attempts to cultivate vaccine virus in extracts of chick embryo tissue. Cultures were incubated at 37°C. Flasks were closed with rubber stoppers. Transfers were made at intervals of 3 or 4 days. The virus underwent a tenfold dilution at the time of each transfer.

— indicates no active virus demonstrable.

TABLE VII

Cultivation of Vaccine Virus in K Medium

Time of titration	Titer of virus in K medium		Titer of virus in meat infusion broth		Titer of virus in Tyrode's solution		Titer of virus in medium containing bits of minced chick embryo tissue
	30°C.	37°C.	30°C.	37°C.	30°C.	37°C.	37°C.
Before incubation	50,000	50,000	50,000	50,000	50,000	50,000	50,000
1st culture	100,000	—	10,000	10,000	100	—	100,000
2nd culture	10	—	>100	>100	—	—	1,000,000
3rd culture	—	—	100	Undiluted	—	—	100,000
4th culture	—	—	Undiluted	—	—	—	100,000

Summary of results obtained in attempts to cultivate vaccine virus in K medium. Meat infusion broth, Tyrode's solution, and minced chick embryo tissue suspended in Tyrode's solution were used as control media. Serial transfers were made at intervals of 3 or 4 days and at the time these were made the virus underwent a twentyfold dilution. Half of the cultures were incubated at 30°C., the other half at 37°C.

— indicates no active virus demonstrable.

K Medium

Repeated failures to cultivate vaccine virus in a "cell-free" medium of the nature described by Eagles and McClean (1, 2) caused us to forego further attempts to induce the active agent to multiply in extracts of tissues. In view of Kendall's (3) suggestion that K medium is suitable for the growth of viruses, we proceeded to determine whether the suggestion is of value in regard to the virus of vaccinia, an active agent that we have for several years been successfully cultivating *in vitro* in the presence of surviving tissue suspended in Tyrode's solution or kept in a mixture of serum and Tyrode's solution. To this end several experiments of the nature of the one described below were performed.

The K medium used in the experiments was supplied by Dr. R. W. G. Wyckoff and was prepared according to Kendall's directions.

Similar amounts of Tyrode's solution in which bits of minced chick embryo tissue were suspended, K medium, Tyrode's solution, and meat infusion broth were inoculated with vaccine virus and placed in amounts of 2 cc. in Carrel D flasks. The flasks were closed with gauze plugs. All cultures were made in duplicate, one set of which was incubated at 30°C., the other at 37°C. At intervals of 3 or 4 days, serial transfers of the cultures were made to fresh media and the titer of the virus in each culture was established by animal experimentation. The results of the experiment are shown in Table VII.

The results of the experiment detailed above and summarized in Table VII clearly indicate that the vaccine virus multiplied in the medium containing bits of living tissue, while no evidence of multiplication of the active agent was found in any of the other media. In fact, the active agent was not demonstrable in Tyrode's solution or in K medium after the second transfers. We were particularly interested to find that the virus survived longer, 4 transfers at 30°C., in the meat infusion broth than it did in the K medium. From the results of our work as well as from an analysis of Kendall's findings there is no reason to suppose that the K medium is capable of supporting the multiplication of vaccine virus or that its use will throw new light upon the nature of this incitant of disease.

DISCUSSION

The results of the experiments described in the first part of the present communication serve as further evidence that vaccine virus is

capable of increasing *in vitro* in the presence of surviving or growing susceptible tissues suspended in a mixture of serum and Tyrode's solution. From an analysis of the findings it becomes obvious that remarkably minute amounts of tissue—5 to 10 mg. of tissue for each 2 cc. of fluid—are adequate. It is not unreasonable to suppose that smaller amounts of tissue may prove to be sufficient.

We were able to cultivate vaccine virus in a medium containing bits of minced spleen from half-grown rabbits. This result is contrary to the findings of Maitland and his coworkers (7, 8) who have stated that they failed to obtain multiplication of the virus in the presence of splenic tissue suspended in a mixture of serum and Tyrode's solution. There is no obvious explanation for this discrepancy unless it can be accounted for on the basis that Maitland and his coworkers may have used too great a concentration of the tissue. There is undoubtedly a tendency for too much tissue to be used in this type of work (11). Furthermore, evidence is lacking to support the belief that similar amounts of different tissues serve equally well for the cultivation of vaccine virus.

Our findings, particularly those dealing with the growth of vaccine virus in "kidney extracts" confirm those of Maitland, Laing, and Lyth (8) but fail to conform to those of Eagles and McClean (1, 2). We, as well as Maitland and his coworkers, have as yet been unable to induce vaccine virus to multiply in the absence of surviving susceptible cells. It appears, therefore, that Eagles and McClean must adduce further evidence before the cultivation of vaccine virus in a "cell-free" medium can be accepted as a fact.

SUMMARY

No evidence was obtained to indicate that vaccine virus is capable of multiplication in media consisting of serum, Tyrode's solution, and extracts of tissues.

Kendall's K medium is not suitable for the cultivation of vaccine virus.

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