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PITUITARY EXTRACTS AND THE VIRUS OF FOOT-AND-MOUTH DISEASE—THE EFFECT ON THE VIRUS OF CERTAIN CHEMICAL METHODS EMPLOYED IN THEIR PREPARATION[1]

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THE experiments recorded here were initiated following upon the occurrence in this country of foot-and-mouth disease in a cow, which a short time previously had been injected for the purpose of inducing oestrus with a pituitary extract imported from the Continent. Mr D. A. E. Cabot, M.R.C.V.S., Chief Veterinary Officer of the Ministry of Agriculture, made reference to this interesting case in a discussion on foot-and-mouth disease at the First Imperial Veterinary Conference held in London (1938). Confirmation of the nature of the infection was obtained by the inoculation of test animals under experimental conditions with suitable material collected from the injected animal. Further investigations were made. Three ampoules containing pituitary extract were obtained from the distributors in London of the imported commercial preparation under discussion. These ampoules belonged to the same batch as the ampoule with the extract from which the cow had been inoculated. Messrs A. Eccles, M.R.C.V.S. and A. M. Graham, M.R.C.V.S., at the Experimental Station of the Foot-and-Mouth Disease Research Committee at Pirbright, tested this material for virus infectivity by the inoculation of cattle and guinea-pigs. They reported that all three samples of pituitary extract were contaminated with the virus of foot-and-mouth disease.[2] In addition they produced evidence that the immunological type of the virus recovered from the extract in the ampoules was the same as that of the virus recovered from the cow, viz. Vallée and Carré O. This and additional information left no doubt that the outbreak of the disease had been produced by the inoculation of the pituitary extract, and further that this extract had been prepared from the pituitary glands of cattle, which were very probably in the early stages of infection with foot-and-mouth disease at the time of slaughter.

It is known that biological products, e.g. calf lymph used for vaccination against smallpox and also swine fever virus and antiserum, have on occasion been responsible for the dissemination of foot-and-mouth disease. This, however, appears to be the first instance in which a glandular extract has proved to

[1] This work was done on behalf of the Foot-and-Mouth Disease Research Committee, who have kindly given their permission for the publication of this paper.

[2] A full report of these experiments will be given in the *6th Progress Report of the Foot-and-Mouth Disease Research Committee* which is now in course of preparation.

be contaminated with the virus under discussion. It is also the first time that the source of an outbreak of foot-and-mouth disease in this country has been traced to the use of any therapeutic substance. This occurrence has shown the need of providing measures for controlling the distribution of such preparations and the Ministry of Agriculture and Fisheries has made recently an order entitled The Foot-and-Mouth Disease (Sera and Glandular Products) Order of 1939 which will come into operation on 10 December 1939. It has been deemed advisable also to institute enquiries as to the methods commonly employed in the preparation of hormones from pituitary glands. Where any doubt existed as to whether any particular chemical method employed would render the product non-infective if virus-contaminated pituitary glands had possibly been employed, it was decided that experiments should be carried out to study the problem. The basic information obtained, while it could not always be considered as conclusive, would be extremely useful in giving some indication of the methods which might yield preparations which would have to be regarded with suspicion as possible sources of virus spread. These observations would be of considerable value, especially at this time, when the question of establishing international standard preparations of prolactin and of the thyrotropic hormone is under discussion, and since it has become necessary to institute legislative measures for the control of the distribution of certain therapeutic substances.

It soon became apparent on enquiry that a considerable number of different methods are employed for the preparation of extracts of the anterior lobe tissue of the pituitary glands; prolactin, thyrotropic hormone, gonadotrophic hormone, and the glycotropic (anti-insulin) principle. Further, as researches on these active principles are in a state of flux, the methods now being employed are liable to modification from time to time. While information has been placed at our disposal by the workers at this Institute as to the main methods commonly employed by them, no assurance can be given that other investigators or manufacturers of therapeutic products would employ exactly similar procedures.

It is obvious that crude extracts of the anterior lobe tissue of the pituitary glands which are prepared by relatively simple procedures, such as aqueous extraction followed by precipitation with a highly concentrated solution of a salt, such as sodium or ammonium sulphate, would have to be regarded with great suspicion. It is known that the virus of foot-and-mouth disease can survive in media containing a high concentration of certain salts, even in saturated solutions of NaCl, Na_2SO_4, $MgSO_4$, etc. (Stockman & Minett, 1926; Pyl & Klenk, 1936; Schlesinger & Galloway, 1937). Pyl & Klenk (1936) showed also that the survival of the virus was considerably prolonged by the presence of NaCl in high concentration, especially when the protein content of the suspending medium was increased, even although the *p*H value was lowered from 7·4 to 6·5, owing to the high salt content. Minett (1928) pointed out also that, although the virus is rendered non-infective in simple media at

*p*H 6·0, it is not completely destroyed by concentrations of acid salts, e.g. $NaHSO_4$, which give hydrogen-ion concentrations higher than this. Edwards & Skinner (1939) (personal communication) have made recently similar observations with regard to virus suspended in media containing high concentrations of NaCl, i.e. some virus may survive under such conditions in media of high hydrogen-ion concentrations, e.g. *p*H 4·0–6·0, unfavourable to its survival in simple buffered media. These have been confirmed by the present writer.

It is understood that the pituitary extract (referred to above) responsible for the infection of a cow with the virus of foot-and-mouth disease had been prepared by aqueous extraction and subsequent precipitation with a certain concentration of sodium sulphate followed by dialysis and drying in a Flosdorf-Mudd apparatus, i.e. at a low temperature. The question of whether virus would always survive this method of treatment would depend on a number of factors. One would expect the dialysis to be continued until all the sodium sulphate was removed, i.e. until a sensitive test for this salt, like the barium chloride test, gave a negative result. When this stage was reached all salts would be removed and there would be a tendency for the hydrogen-ion concentration of the extract to approach the isoelectric point of the common tissue proteins, i.e. in the region of *p*H 5·0–5·5. It is known that this reaction is lethal to the virus in simple media under ordinary conditions (cf. in the presence of high concentration of certain salts, *vide supra*). However, it might well be that if time saving were considered to be of importance dialysis might not be carried so far. It is possible also that other proteins or tissue substances present in the extract might prevent the hydrogen-ion concentration becoming as high as this and virus would be more likely to survive. The final drying process at low temperature would, of course, favour the survival of the virus. It was considered to be unnecessary to make any experiments with this method, in view of the fact that it had apparently in one case at least yielded an infective product and there were too many possible variables to control.

It is obviously impossible to investigate all the methods which are used or might possibly be used in making pituitary extracts. Attention has, therefore, been directed to a study of some of the basic methods employed in the preparation of extracts of anterior lobe tissue, such as (1) "acetone desiccation", (2) preparation of crude prolactin by alkaline extraction in the cold followed by precipitation and reprecipitation at *p*H 5·5 and separation of the crude thyrotropic fraction, (3) preparation of crude whole extract of anterior lobe (B fraction), which involves repeated extraction of "acetone-desiccated" gland at room temperature with 60 % alcohol (*p*H adjusted to 9–10), subsequent precipitation at *p*H 5 and the addition of absolute alcohol and drying by ether and *in vacuo* over $CaCl_2$.

No prediction could be made as to the possible issue in these experiments, with the exclusion, perhaps, of the "acetone desiccation" method. It has been found in other experiments that the virus even in filtrates can resist the action of 50, 60, 70 and even 80 % acetone (neutral) at 0° C. for at least 24 hr. While

from a superficial consideration of the processes, it might be assumed that the several chemical treatments employed would certainly be lethal to the virus, it must be borne in mind that there are a number of factors which, on the other hand, would tend to favour survival of the virus, e.g. methods (1) and (2) (*vide supra*) are generally carried out almost entirely in the cold room at $-2°$ to 0° C., because the hormones are believed to be labile structures, and the presence of a large amount of tissue substances in the extracts would tend to have a protective action on the virus in counteracting effects such as change in hydrogen-ion concentration and the lethal effect of certain concentrations of alcohol, which in simple liquids without tissue would inactivate the virus.

In all the experiments recorded below, the strain of virus employed was "Rouen", a strain recently recovered from cattle during the recent epizootic in France in 1937. This strain had undergone only about thirty passages in guinea-pigs at the time the experiments were commenced. Stock broth filtrates of virus were prepared for each group of experiments carried out within the same period of 10–15 days, and the average titre (limiting infective dilution) of all filtrates was about 10^{-5}. In some preliminary experiments the degree of contamination of the anterior lobe tissue (15–20 g.) with virus was not as great as in the later experiments in which a relatively high concentration was present in the tissue suspensions.

Notes on the virus content of pituitary glands

There is no information available as to the virus content of pituitary glands removed at different stages from cattle, sheep and pigs infected with foot-and-mouth disease, and in studies on hormones, pituitary extracts are prepared from the glands from all these three species and also from horses. Investigations on this point have been planned. A certain number of preliminary tests have been made on guinea-pigs. The pituitary glands were ablated from five groups of six guinea-pigs which had been infected intradermally with the virus of foot-and-mouth disease and were showing generalized lesions. The first batch of glands was removed at about the 48th hour, i.e. when generalized lesions were first observed, and the last on the 6th day. Each gland was ground in a mortar with 1 c.c. of buffered phosphate solution at *p*H 7·6 and samples from the suspension were injected into the pads of two guinea-pigs intradermally. Virus was recovered from three of six of the glands removed at about the 48th hour, two of six removed about the 72nd hour, but none was detected in any of the total of eighteen glands tested on the 4th, 5th and 6th days. These results, which are recorded in Table I, would suggest that the period of infectivity of the pituitary bodies coincides with what previous experience has shown is the period of infectivity of the blood. No histological study of the pituitaries taken from infected guinea-pigs has yet been made, but this will be done to find out whether any departure from normality can be detected. Similar investigations will be made on pituitary glands from infected cattle, pigs and sheep, whenever this may be possible.

Table I. *Testing of pituitary glands from infected guinea-pigs for virus content*

Time after infection	No. of guinea-pig	Generalized lesions	Presence of virus in suspension		Total
			1	2	
48 hr.	1	+	0	0	
	2	+	+ +	+ +	
	3	+	+ +	0	3/6
	4	+	0	0	
	5	+	+ +	+ +	
	6	+	0	0	
3 days	7	+	0	0	
	8	+	0	0	
	9	+	+ +	0	2/6
	10	+	0	0	
	11	+	0	0	
	12	+	+ +	+ +	
4 days	13	+	0	0	
	14	+	0	0	
	15	+	0	0	0/6
	16	+	0	0	
	17	+	0	0	
	18	+	0	0	
5 days	19	+	0	0	
	20	+	0	0	
	21	+	0	0	0/6
	22	+	0	0	
	23	+	0	0	
	24	+	0	0	
6 days	25	+	0	0	
	26	+	0	0	
	27	+	0	0	
	28	+	0	0	0/6
	29	+	0	0	
	30	+	0	0	
	31	+	0	0	

+ + = generalized lesions in inoculated guinea-pigs.
0 = no reactions in inoculated guinea-pigs.
Number of guinea-pigs for each test = 2.

Even if in these latter species no evidence be obtained that the virus multiplies and produces lesions in these glands, but on the contrary that the virus content is due to the blood present, there are other means of contamination of the pituitaries to be considered. In the process of removal of the pituitary glands at the slaughter house, obviously chopping of the surrounding bone and tissues is carried out. Many of the pituitaries received here have portions of the sphenoid bone attached, and it is probable that small pieces of bone marrow and even mucus and epithelium from the nasal fossae and sinuses may become mixed with the gland tissue. If the bone marrow, etc., happened to contain virus, virus contamination of the pituitaries might occur in this way and at a time when the virus was no longer circulating in the blood.

Since no estimate can be made of the amount of virus which the pituitaries would be likely to contain, large quanta of virus have been mixed with the frozen pituitaries employed in the experiments about to be described, to make the tests as severe as possible. It will be realized of course that the virus added

to gland tissue in this way may not penetrate the cells in the same way as it does the epithelial cells of certain regions of the infected animal. Experience has already shown that virus in suspensions of infected cells is much more resistant to conditions which would be harmful to "free" virus, e.g. the writer has found (Galloway, 1931) that whereas "free" virus suspended in buffered phosphate saline at *p*H 7·5 was rapidly inactivated by the change in the reaction of the medium when the suspension was frozen (crystallizing out of alkaline phosphate), virus in an emulsion of the skin of the plantar pads was not so affected. This must be borne in mind in a consideration of the results of the following experiments.

Results of experiments

(1) *Experiments on the effect of "acetone desiccation" on the infectivity of anterior lobe tissue (pituitary glands) artificially contaminated with virus*

(*a*) Fresh ox pituitary glands were procured from the slaughter-house, and the anterior lobe tissue was dissected out. 15 g. of tissue were employed for each experiment.

(*b*) In group I experiments (see Tables II and III, A, B, C, F and G), the following method was employed of contaminating the pituitaries with virus: 5 c.c. of a broth filtrate of virus (titre about 10^{-5}) were pipetted on to the tissue in a Petri dish. The tissue was then placed at $+4°$ C. for 24 hr. with the idea of facilitating the absorption of the virus by the tissue. The tissue without the unabsorbed virus was then transferred to another clean Petri dish and frozen at $-10°$ C.

Table II. *Test for infectivity of pituitary glands (anterior lobes) artificially contaminated with virus and subsequently dried* in vacuo *over $CaCl_2$ after extraction with ice-cold dry acetone alone or ice-cold dry acetone followed by cold alcohol and cold dry ether*

Group I experiments. Temperature $-2°$ to $0°$ C.

Control of contaminated gland suspension Guinea-pigs		Batch	Treatment	Test for virus Guinea-pigs			
1	2			1	2	3	4
+ +	0	A	20 vol. ice-cold acetone 24 hr. Desiccation *in vacuo* $CaCl_2$	+ +	0	+ +	0
+ +	+ +	B	20 vol. ice-cold acetone followed by washing with cold absolute alcohol and cold dry ether on a filter paper. Desiccation *in vacuo* $CaCl_2$	+ +	0	0	0
+ +	0	C	20 vol. ice-cold acetone 24 hr.; 20 vol. ice-cold acetone 24 hr.; 20 vol. ice-cold acetone 24 hr. Desiccation *in vacuo* $CaCl_2$	+ +	0	+ +	0
+ +	+ +	F	20 vol. ice-cold acetone 24 hr. Desiccation *in vacuo* $CaCl_2$	+ +	+ +	0	0
+ +	+ +	G	20 vol. ice-cold acetone 24 hr. followed by washing with cold absolute alcohol and cold dry ether on a filter paper. Desiccation *in vacuo* $CaCl_2$	+ +	+ +	0	0

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Table III. *Retest of "acetone-desiccated" pituitary glands (anterior lobes) from group 1 experiments (see Table II) after storage at −2° to 0° C. and 18° to 20° C. in stoppered jars*

Group I experiments

Batch	Time of storage days	Temperature ° C.	Test for virus Guinea-pigs 1	2	3	4
A	50	18 to 20	0	0	0	0
A	50	−2 to 0	0	0	0	0
B	50	18 to 20	0	0	0	0
B	50	−2 to 0	0	0	0	0
C	50	18 to 20	0	0	0	0
C	50	−2 to 0	0	0	0	0
F	44	18 to 20	0	0	0	0
F	44	−2 to 0	0	0	0	0
G	44	18 to 20	0	0	0	0
G	44	−2 to 0	+ +	0	0	0

+ + = generalized lesions in inoculated guinea-pigs.
0 = no reactions in inoculated guinea-pigs.

In group II experiments (Tables IV and V, L, M, P) the degree of contamination of the anterior lobes of the pituitary glands was probably much greater. 3 c.c. of a broth filtrate of virus were pipetted on to the glands placed in a Petri dish, and after 24 hr. at 4° C. they were not transferred to a fresh dish but the tissue and the virus were frozen together.

Table IV. *Test for infectivity of anterior lobe tissue artificially contaminated with virus and subsequently dried* in vacuo *over $CaCl_2$ after extraction with ice-cold dry acetone alone or ice-cold dry acetone followed by cold alcohol and cold dry ether*

Group II experiments. Temperature −2° to 0° C.

Control of contaminated gland suspension Guinea-pigs 1	2	Batch	Treatment	Test for virus Guinea-pigs 1	2
+ +	+ +	L	20 vol. ice-cold acetone 24 hr. followed by washing with cold absolute alcohol and cold dry ether on a filter paper. Desiccation *in vacuo* $CaCl_2$	+ +	+ +
+ +	+ +	M	As above	+ +	+ +
+ +	+ +	P	As above	+ +	+ +

+ + = generalized lesions in inoculated guinea-pigs.

(*c*) All subsequent steps in the process were carried out at −2° to 0° C. The frozen virus-contaminated anterior lobes were ground in a mortar and twenty volumes of ice-cold acetone added. The tissue remained in the acetone overnight. Subsequently, one of the three following procedures was adopted:

(c_1) (Batches A and F, Table II). The acetone was decanted and replaced by a fresh quantum of twenty volumes of ice-cold acetone. The flask was shaken thoroughly and the acetone filtered off. The tissue extract was scraped into a Petri dish and dried over $CaCl_2$ *in vacuo*.

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Table V. *Retest of "acetone-desiccated" anterior lobe tissue of pituitary glands from group II experiments (see Table IV) after storage under different conditions*

Group II experiments

No. of test	Batch	Method of storage	Time of storage	Temperature ° C.	Test for virus Guinea-pigs 1	2	3	4
1	L	In test-tube plugged with cotton-wool	18 days	−2 to 0	+ +	+ +	+ +	0
2	L	Do.	Further 7 days	18 to 20	+ +	+ +	.	.
1	$M^{(A)}$	Do.	14 days (17 days since preparation)	−2 to 0	+ +	+ +	+ +	+ +
2	$M^{(A)}$	Do.	Further 7 days (24 days since preparation)	18 to 20	+ +	0	.	.
1	$M^{(B)}$	In sealed test-tube	14 days (17 days since preparation)	−2 to 0	+ +	+ +	+ +	+ +
1	$M^{(C)}$	Do.	14 days (17 days since preparation)	18 to 20	+ +	+ +	+ +	+ +
1	$M^{(D)}$	*In vacuo*	17 days	−2 to 0	+ +	+ +	+ +	+ +
2	$M^{(D)}$	Later in test-tube plugged with cotton-wool	Further 7 days (24 days since preparation)	18 to 20	+ +	0	0	0
1	$P^{(A)}$	In test-tube plugged with cotton-wool	7 days	18 to 20	+ +	0	.	.
1	$P^{(B)}$	Do.	7 days	18 to 20	+ +	0	0	0

+ + = generalized lesions in inoculated guinea-pigs.
0 = no reactions in inoculated guinea-pigs.

(c_2) (Batch C, Table II). The tissue after remaining in the acetone overnight, was transferred at intervals of 24 hr. to two fresh 20-volume amounts of ice-cold acetone. The acetone was then filtered off as in (c_1) and the extract dried *in vacuo*.

(c_3) (Batches L, M and P, Table IV, and B and G, Table II). After remaining in the acetone overnight, the tissue was washed several times on a filter paper with absolute alcohol and dry ether before being dried in the desiccator.

(*d*) Subsequently the different batches of "acetone-desiccated" anterior lobe tissue were tested for infectivity. A small sample of about 0·5 g. was ground in 1 c.c. of buffered phosphate solution at *p*H 7·6. Two to four guinea-pigs were inoculated intradermally into the pads with the suspensions. The results are recorded in Tables II and IV. All the batches of tissue desiccated by acetone alone; three changes of acetone; or acetone alcohol and ether, proved to be infective to some degree.

The different batches of "acetone-desiccated" anterior lobe tissue were stored for varying periods under different conditions. Only one of the samples tested from batches A, B, C, F and G (see Table III) proved to be infective when tested after storage. This sample (G) had been in a stoppered bottle at −2° to 0° C. for 44 days.

Batches L, M and P (Table V) were stored either at 18–20° C., or −2 to 0° C. under different conditions. The results show that the virus survived for a period of at least 18 days at 0° C. in test-tubes plugged with cotton-wool. Two samples kept in the same way remained infective for at least 7 days at 18–20° C.

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Samples kept in sealed test-tubes remained infective for at least 14 days at either 18–20° C. or −2° to 0° C. A sample kept *in vacuo* at 0° C. for 17 days and subsequently at 18–20° C. for 7 days in a test-tube plugged with cotton-wool also remained infective. In the case of four of the samples, $M^{(A)}$, $M^{(D)}$, and $P^{(A)}$, $P^{(B)}$ (Table V), kept in test-tubes plugged with cotton-wool for 7 days at 18–20° C. there was suggestive evidence that the virus infectivity had diminished.

"Acetone-desiccated" anterior lobe tissue is often used as "starting" material for the preparation of different fractions or extracts of the anterior pituitary tissue, and in view of the observations recorded here, it must be regarded as dangerous, when there is a possibility of the pituitary glands having originated from an infected animal.

The results of these experiments show that virus will survive any of the procedures likely to be employed in its preparation: e.g. (1) extraction and dehydration with acetone at 0° C. during one period of 24 hr.; (2) extraction and dehydration at 0° C. with two changes of acetone at 24 hr. intervals; or (3) extraction with acetone followed by dehydration with absolute alcohol and ether at 0° C.

The period for which the "acetone-desiccated" tissue may remain infective will depend on a number of factors, such as the original amount of virus present and the method of storage. The dried anterior lobe would probably remain infective for longer periods if stored in bulk than in small amounts. The questions of exposure to air and temperature would also have an influence. Low temperature and *in vacuo* storage would favour survival. Samples stored in stoppered bottles or sealed tubes would remain infective longer than if exposed to the air. In the present experiments one batch of desiccated anterior lobe was infective after 44 days at 0° C. in a stoppered bottle. Other batches remained infective at either room temperature or in the cold for periods of at least 7–24 days (these were not end points) depending on the conditions. The period of survival is determined by the degree of "chemical" dryness.

(2) *Preparation of crude prolactin by alkaline extraction in the cold followed by precipitation and reprecipitation at* pH 5·5 *and separation of crude thyrotropic fraction*

In these experiments fresh ox anterior lobes of the pituitary gland were contaminated with the virus in the same way as in the series of experiments just described. However, only in Exp. 1 (group I), Table VII, was the pituitary gland tissue contaminated with virus by the method employed in group I experiments of the preceding section. In the remaining or group II experiments, 3 c.c. of a broth virus filtrate (titre about 10^{-5}) were poured over and frozen with the 15 g. amounts of tissue. All the procedures in this method were carried out at −2° to 0° C. including the centrifugation, during the processes of precipitation and reprecipitation. The method was originally described by Young (1938). The frozen virus-contaminated anterior lobes of the pituitaries

were minced and ground with sand in a mortar with addition of 3 ml./g. of cold saline. The *p*H was adjusted to 8·5 by addition of *N*/5 NaOH, employing thymol blue as the colour indicator. As will be seen from the results of the tests recorded in Table VI, nine extracts prepared in this way were still infec-

Table VI. *Testing of infectivity of alkaline extracts of pituitary glands (anterior lobes). (Fresh glands contaminated with virus then frozen-ground with sand and 3 ml./g. cold saline pH adjusted to 8·5)*

Temperature −2° to 0° C.

	Test of contaminated glands for virus		Test of extract		
Extract	(1)	(2)	(1)	(2)	Storage period
D	+ +	+ +	+ +	+ +	24 hr.
E	+ +	+ +	+ +	+ +	24 hr.
H	—	—	+ +	+ +	24 hr.
I	—	—	+ +	+ +	24 hr.
J	—	—	+ +	+ +	24 hr.
			Retest + +	+ +	19 days
K	—	—	+ +	+ +	24 hr.
			Retest + +	+ +	19 days
N	—	—	+ +	+ +	24 hr.
O	—	—	+ +	+ +	24 hr.
Q	—	—	+ +	+ +	24 hr.

+ + = generalized lesions in inoculated guinea-pigs.
(1) (2) = two test animals.

tive, and in two cases it was shown that some degree of infectivity remained in such extracts for at least 19 days at 0° C. The extract was left in the cold overnight, when the hydrogen-ion concentration was again readjusted, if necessary, to *p*H 8·5. The suspension was then centrifuged for 15 min. at about 2000 rev./min. The thick extract was decanted and the residue discarded. The hydrogen-ion concentration was adjusted to *p*H 5·5 (chloro-phenol red as colour indicator) with *N*/5 HCl, and again centrifuged. The clear supernatant was pipetted off. The precipitate was stirred with a volume of cold saline equal to the original amount used for extraction, and the hydrogen-ion concentration readjusted to *p*H 8·5. After 30 min. extraction the reaction was adjusted to *p*H 5·5 and the mixture again centrifuged.

Crude prolactin fraction. Four precipitations were effected in this way in Exp. 1 (this is exactly as the method is carried out in the preparation of prolactin). The prolactin was then stirred with a volume of cold saline, equal to the original amount used for extraction, at *p*H 8·0.

Crude thyrotropic fraction. The clear supernatant fluid from the first precipitation at *p*H 5·5 was made up to a volume equal to the final volume of the prolactin fraction by addition of a part of the supernatant fluid from the second prolactin precipitation and fresh saline, the hydrogen-ion concentration being adjusted to *p*H 8·0.

Results. In Exp. 1 a sample of the extract from the first precipitation was tested on two guinea-pigs by intradermal pad inoculation and the crude prolactin and the crude thyrotropic fraction on four guinea-pigs. No reactions

occurred in any of these animals, indicating that the virus present in the alkaline extract had been rendered non-infective by the effect of the precipitation at *p*H 5·5 (dilution probably also played a part).

It was then decided to repeat the experiments while limiting the tests to the alkaline extract, the first precipitate (*p*H 5·5) and the crude thyrotropic fraction. The results of four experiments made in this way (2–5) are recorded in Table VII. In two of these experiments (one of four guinea-pigs reacted in each

Table VII. *Testing of infectivity of different fractions obtained during the preparation of crude prolactin and the crude thyrotropic fraction (starting material, fresh frozen virus-contaminated glands)*

Brief summary of method: Alkaline extraction (*p*H 8·5) of anterior lobe tissue from pituitary glands at 0° C. followed by precipitation and reprecipitation of the prolactin at *p*H 5·5—the supernatants from the 1st and 2nd precipitations constituting the crude thyrotropic fraction (readjusted finally to *p*H 8·0).

Exp.	Alkaline extract	Tests for virus content of different fractions: 1st ppt. *p*H 5·5 (1) (2) (3) (4)	Crude prolactin (4 ppt. *p*H 5·5) (1) (2) (3) (4)	Crude thyrotropic fraction *p*H 8·0 (1) (2) (3) (4)
1*	(D) + + (group 1)	0 0	0 0 0 0	0 0 0 0
2	(H) + + (group 2)	0 0 0 0	—	—
3	(I) + + (group 2)	+ + 0 0 0	—	—
4	(J) + + (group 2)	+ + 0 0 0	—	0 0 0 0
5	(K) + + (group 2)	+ + 0 0 0	—	0 0 0 0
6*	(J) + + (group 2)	0 0 0 0	—	0 0 0 0
7*	(K) + + (group 2)	0 0 0 0	—	0 0 0 0
8*	(S) + + (group 2)	0 0 0 0	—	0 0 0 0
		2 ppt.	3 ppt.	Crude thyrotropic (*p*H 8) fraction
9	(N) + + (group 2)	0 0 0 0	—	0 0 0 0
10	(O) + + (group 2)	0 0 0 0	—	0 0 0 0
11	(Q) + + (group 2)	0 0 0 0	0 0 0 0	0 0 0 0
12	(R) + + (group 2)	0 0 0 0	0 0 0 0	0 0 0 0

* Precipitation overnight in cold.

+ + = generalized lesions in inoculated guinea-pigs.

0 = no reactions in inoculated guinea-pigs.

(1) (2) (3) (4) = four guinea-pigs.

case) some virus had survived the first precipitation. The tests were made on samples removed 30 min. after the reaction had been adjusted to *p*H 5·5 (centrifugation was carried out 20 min. after the adjustment of the *p*H). In Exps. 6–8 the precipitation was allowed to proceed in the cold overnight and after centrifugation the first precipitate was tested on four guinea-pigs, but none developed lesions. The crude thyrotropic fraction (supernatant from first precipitation only) was tested in Exps. 4–8 with negative results.

Further experiments were made (9–12, Table VII). In four experiments the extract was tested on four guinea-pigs after two precipitations and in two experiments after three precipitations, with entirely negative results. The crude thyrotropic fractions in Exps. 9–12 produced no reaction in any of the four guinea-pigs inoculated with the respective samples.

Thus alkaline extracts (*p*H 8·5) made from anterior lobe tissue artificially contaminated with virus, may remain infective at 0° C. for at least 19 days.

During the process of preparation of crude prolactin, which involves precipitation and reprecipitation in the cold at *p*H 5·5, the extracts became non-infective. The virus present in the alkaline extracts could, however, survive one precipitation at *p*H 5·5 if the time interval was short (about 30 min.). When precipitation was allowed to proceed slowly (24 hr. in cold) the infectivity of the extract was removed (tests on four guinea-pigs). The time factor therefore seems to be important. Even if efficient mixing is carried out, it would not be certain that the *p*H value 5·5 was attained almost immediately throughout the mixture, and of course if the virus were inside epithelial cells, for example, the chance of survival would be increased. Two precipitations at *p*H 5·5 gave negative results in tests for virus in four experiments, and three precipitations at *p*H 5·5 negatives in two experiments. The crude thyrotropic fractions were tested in ten experiments and none proved to be infective.

The results of the tests would suggest that crude prolactin or crude thyrotropic substance prepared by the method of precipitation and reprecipitation at *p*H 5·5 from alkaline extracts of virus-contaminated pituitary glands, are most likely to be non-infective. However, if the virus did multiply inside the cells of the pituitary gland of cattle, pigs or sheep, or if the glands became contaminated with mucus containing desquamated epithelial cells during the process of their removal, the possibility of the survival of the virus under certain conditions cannot be excluded with certainty.

(3) *Preparation of crude whole extract of anterior lobe (B fraction) (including tests on A fraction not used at present by workers on hormones)*

In these experiments the method employed was as follows:

Infective "acetone-desiccated" anterior lobe tissue (see L, M, P, Table IV) were extracted for 1 hr. at 18–20° C. with ten volumes of 60 % alcohol. The *p*H of the mixture was adjusted to about 9–10 by the addition of *N*/5 NaOH—employing phenolphthalein as the colour indicator. The insoluble fraction was centrifuged off, and further extraction (three times) of this insoluble fraction was carried out with 60 % alcohol (*p*H 9–10). (The insoluble fraction after extraction is designated A fraction, and although it has been tested for infectivity in the present experiments, its possible properties have not yet been considered by the chemists and physiologists.) The three supernatants after centrifugation were adjusted to about *p*H 5 (indicator methyl red) and three volumes of absolute alcohol were added. The precipitate which formed (flask stored at 0° C. for 24 hr.) was collected and dried by alcohol and ether and *in vacuo* over $CaCl_2$ at 0° C. This dried material constitutes the crude whole extract of anterior lobe, B fraction. In this method it will be observed that all the processes involving extraction, adjustment of hydrogen-ion concentration, centrifugation, etc., were carried out at room temperature.

The A fraction was also dried over $CaCl_2$. Samples of A fraction and B fraction were suspended in a small quantity of buffered phosphate saline at *p*H 7·6 and tested for infectivity by inoculation intradermally into four guinea-

pigs. The results of three experiments are recorded in Table VIII. None of the three samples of B fractions or the three samples of A fractions tested proved to be infective.

Table VIII. *Testing of infectivity of B fraction (crude whole extract of anterior lobe) and A fraction (insoluble fraction) separated from "acetone-desiccated" glands contaminated with virus*

Brief summary of method: "Acetone-desiccated" gland extracted for 1 hr. at 18–20° C. with 10 volumes 60% alcohol (*p*H adjusted to 9–10)—insoluble fraction centrifuged off and further extraction carried out. Insoluble fraction = A extract. Supernatants adjusted *p*H 5, 3 volumes of absolute alcohol added, precipitate collected, dried with alcohol, ether and *in vacuo*.

No. of experiment and stock desiccated gland	Control of infectivity of desiccated glands at time of making experiment		Test of B fraction (dried)				Test of A fraction (dried)			
	1	2	1	2	3	4	1	2	3	4
Exp. 1 L (see Table V)	+ +	+ +	0	0	0	0	0	0	0	0
Exp. 2 M (see Table V)	+ +	+ +	0	0	0	0	0	0	0	0
Exp. 3 P (see Table V)	+ +	+ +	0	0	0	0	0	0	0	0

Figures indicate no. of guinea-pigs.
+ + = generalized lesions.
0 = no reaction.

Effect of 60% alcohol alone on infectivity of acetone-desiccated virus-contaminated anterior lobe tissue. When the results of these experiments were obtained it was decided to investigate an intermediate stage in the preparation of crude anterior lobe extract (B fraction), viz. the effect of 60% alcohol on the infectivity of "acetone-desiccated" virus-contaminated lobe tissue. Five experiments were carried out. In three of these (1–3, Table IX), 1 g. of infected gland tissue was ground with ten volumes of 60% alcohol in a mortar, and at intervals samples were removed and inoculated into the pads of four guinea-pigs. No reactions occurred in any of the thirty-six guinea-pigs inoculated with the different samples removed after 10 min., 20 min. and 1 hr. In Exp. 4, "acetone-desiccated" virus-contaminated gland was ground in ten volumes of 60% alcohol and left in contact for 15 min. The mixture was then centrifuged for 5 min. and the deposit was resuspended in a small quantity of buffered phosphate solution at *p*H 7·6 before inoculation into four guinea-pigs. None of these animals showed any reactions.

Exp. 5 was carried out in the same way as Exp. 4, and at the same time the effect of absolute alcohol was tested under the same conditions. None of the four guinea-pigs inoculated with the suspensions of extracts treated with 60% alcohol developed lesions, but two out of four guinea-pigs inoculated with the material treated with absolute alcohol developed lesions. (N.B. The effect of different concentrations of alcohol on the virus of foot-and-mouth disease has been studied by Abe (1925), Bedson & Maitland (1925), Olitsky & Boëz (1927), and Galloway & Elford (1936). The observations made by the different authors

Table IX. *Effect of* 60 % *alcohol and absolute alcohol on infectivity of "acetone-desiccated" glands artificially contaminated with virus*

Batch of "acetone-desiccated" glands employed = P (*vide* Table V). Temperature 18–20° C.

Exp.	Control of infectivity of desiccated glands at time of making experiment	Time of contact with alcohol	Test for virus (1)	(2)	(3)	(4)
1	+ +	10 min.	0	0	0	0
		20 min.	0	0	0	0
		60 min.	0	0	0	0
2	+ +	10 min.	0	0	0	0
		20 min.	0	0	0	0
		60 min.	0	0	0	0
3	+ +	10 min.	0	0	0	0
		20 min.	0	0	0	0
		60 min.	0	0	0	0
4	+ +	Desiccated gland ground in mortar with 60 % alcohol left 15 min. centrifugalized 5 min. deposit resuspended in buffered phosphate after removal of alcohol	0	0	0	0
5	+ +	Do.	0	0	0	0
		Desiccated gland ground in mortar with absolute alcohol then treated as above	+ +	+ +	0	0

+ + = generalized lesions in inoculated guinea-pigs.
0 = no reactions in inoculated guinea-pigs.
(1) (2) (3) (4) = four test animals.

under varying conditions have been summarized by the last-named authors. From a result of their own experiments—made under standard conditions, Galloway & Elford arrived at the conclusions that the degree of the resistance of the virus to 60 % alcohol is much less than has been generally supposed, and that it can be explained in part by the presence of serum proteins with which it is associated in unpurified preparations. Unpurified virus behaved very similarly, however, to purified virus at 22° C.—both survived for 5–15 min., but not 30 min. On the other hand, in the cold at 0 and −10° C. the unpurified virus remained infective in 60 % alcohol for at least 7 days—purified virus was inactive after 4 days at 0° C. and 7 days at −10° C.)

In the present experiments, infective "acetone-desiccated" anterior lobe tissue treated with 60 % alcohol was apparently non-infective after 10–15 min. at 18–20° C. The amount of virus present in such desiccated material is probably not very great. In comparative tests at 18–20° C. absolute alcohol had apparently a less lethal effect than the 60 % alcohol; virus-contaminated desiccated gland was not rendered non-infective in 15 min.

Thus the procedures employed in the preparation of crude whole extract of anterior lobe (B fraction), which include extraction for 1 hr. at 18–20° C. in ten volumes of 60 % alcohol at *p*H 9–10, followed by precipitation at *p*H 5, and addition of absolute alcohol and desiccation by alcohol, ether and then *in vacuo*, were sufficient to inactivate the virus present in the virus-contaminated acetone-desiccated anterior lobe. The experiments on the effect of 60 % alcohol alone showed that the acetone-desiccated anterior lobe tissue was rapidly rendered non-infective by this treatment. At different stages during the course of preparation of the crude whole extract of anterior lobe by this method three

sets of conditions obtain which are known to be harmful to the virus in simple fluid media—suspension in 60% alcohol at room temperature, especially in an alkaline medium (see Olitsky & Boëz, 1927), a hydrogen-ion concentration of 9–10 at room temperature, and a hydrogen-ion concentration of 5 at room temperature. Pituitary extracts made by this method would almost certainly be non-infective. The B fraction may be employed as such or for the preparation of purified hormones.

Effect of infectivity of swinging the hydrogen-ion concentration of suspensions of prolactin in a virus medium (to pH 5, *then to* pH 7·6). These experiments were carried out with the object of determining whether swinging the hydrogen-ion concentration of a suspension of a prolactin preparation, supposed to be infected with the virus of foot-and-mouth disease, would be a possible method of rendering the material non-infective.

Methods. 20 mg. amounts of prolactin LP 5 or LP 7 were suspended (most of the material went into solution) in 2 c.c. of a broth filtrate of virus having a titre of about 10^{-5}. The reaction of the mixtures was adjusted to *p*H 5 with *N*/5 HCl. A precipitate formed, as this *p*H is near the isoelectric point of the prolactin. In each experiment two tubes of such mixtures were prepared. The hydrogen-ion concentration of the mixture in one tube was immediately swung back to *p*H 7·6 with *N*/5 NaOH. The precipitate redissolved when the *p*H was readjusted. The mixture in the other tube was left for 1 hr. at *p*H 5 before the hydrogen-ion concentration was swung back to *p*H 7·6. Samples from each of the tubes were inoculated into each of four guinea-pigs. None of these guinea-pigs showed lesions. Three experiments were made under similar conditions, i.e. at 18–20° C. and no virus infectivity could be detected in the prolactin suspensions, the hydrogen-ion concentrations of which had been swung to *p*H 5 and then back to *p*H 7·6 immediately or after 1 hr. at *p*H 5 (see Table X).

Table X. *Test for effect on infectivity of swinging the hydrogen-ion concentration of suspensions of prolactin in a virus medium (to* pH 5 *then to* pH 7·6)

Preparations employed = LP 5 prolactin, LP 7 prolactin

Exp.	Preparation	Control of suspension of LP 5 in virus		Test for virus (*p*H 5 and swung to *p*H 7·6 immediately)				Test for virus (*p*H 5 for 1 hr. and swung to *p*H 7·6)			
		1	2	1	2	3	4	1	2	3	4
1	LP 5	+ +	+ +	0	0	0	0	0	0	0	0
2	LP 5	+ +	+ +	0	0	0	0	0	0	0	0
3	LP 7	+ +	+ +	0	0	0	0	0	0	0	0

Figures indicate no. of guinea-pigs.
+ + = generalized lesions in inoculated guinea-pigs.
0 = no reactions in inoculated guinea-pigs.

In all these experiments great care was taken to ensure that no virus was splashed on the upper part of the small test-tubes used, and that all the fluid reached the requisite hydrogen-ion concentration. It had previously been observed in experiments of this nature that irregular results will occur if care

is not taken to prevent some virus escaping the effect of the acid reaction. This would be of importance in a consideration of the use of this method of treating hormone preparations which were under suspicion as possible sources of virus infection.

Summary and general conclusions

It was decided to institute enquiries as to the methods commonly employed in the preparation of hormones from pituitary glands following upon the report that evidence had been obtained that an outbreak of foot-and-mouth disease in this country was due to the injection of a cow with a pituitary extract imported from the Continent. It was obviously impossible to investigate all the methods which are used or might possibly be used in making pituitary extracts. It was clear also that there were certain extracts which, having regard to their method of preparation, would have to be considered as possible sources of virus spread if there was any likelihood that they had been made from glands obtained from an infected source. Among these would be classed crude extracts of the anterior lobe tissue of the pituitary glands which are prepared by relatively simple procedures, such as aqueous extraction, which may be followed by precipitation by salting out methods and especially when the extracts are subsequently desiccated.

The investigations reported on here were confined, therefore, to a study of the effect on the virus of foot-and-mouth disease of three chemical methods commonly employed in the preparation of hormones or crude extracts of the pituitary glands. In these experiments fresh anterior lobes of ox pituitary glands were contaminated with the virus of foot-and-mouth disease and used as "starting" material. The results obtained and the general conclusions drawn therefrom are as follows:

(1) *Acetone-desiccation in the cold.* This method may include, in addition to extraction with acetone, treatment with absolute alcohol and ether and involves subsequent desiccation of the extract *in vacuo* over $CaCl_2$. These processes did not inactivate the virus of foot-and-mouth disease in any of eight experiments. How long such desiccated pituitary extracts may remain infective will depend on the amount of virus present and on the method of storage. Any method of storage by which the "water content" of the dried material is kept at a minimum will favour survival. In the present experiments, samples of virus-contaminated "acetone-desiccated" anterior lobe tissue have been found to be infective after periods of 7, 29 and 44 days (not end-points), depending on the conditions of storage. In view of these findings "acetone-desiccated" extracts of pituitary gland must be regarded as dangerous, since they would be a possible source of virus spread if the glandular tissue should have originated from infected animals.

(2) *Preparation of crude prolactic by alkaline extraction in the cold followed by precipitation and reprecipitation at* pH 5·5 *and separation of the crude thyrotropic fraction.* The preliminary stage of this method is the alkaline extraction, pH 8·5, of the anterior lobes of the pituitary glands. Nine extracts prepared in

this way from virus-contaminated glandular tissue maintained their virus infectivity, and in two instances in which the alkaline extracts were tested after storage for a period of 19 days at 0° C. some virus was still detectable. The method usually involves *four* precipitations of prolactin at *p*H 5·5. Three (two experiments) or even two (four experiments) precipitations appeared to be sufficient to remove the virus infectivity (tests on four guinea-pigs) of the prolactin fraction, although one precipitation was not always sufficient when the time interval was short (about 30 min.). The virus did not survive in the prolactin fraction of the first precipitation at *p*H 5·5 when the process was allowed to proceed in the cold for 24 hr. None of the ten thyrotropic fractions tested produced foot-and-mouth disease in guinea-pigs. There is very little possibility of virus surviving this method of preparation of prolactin, even if, as is apparently usually the case, it is carried out in the cold (about 0° C.), unless experimental conditions be such that all the extract does not reach the requisite hydrogen-ion concentration, or the virus happens to be present within epithelial or other cells when it might not be affected by the reaction *p*H 5·5 usually unfavourable to its survival. It is even less likely that the virus would survive in the thyrotropic fraction, which is a clear fluid, the reaction of which reaches *p*H 5·5 during the course of separation, and is later readjusted to *p*H 8·0.

(3) *Preparation of crude whole extract of anterior lobes* (*B fraction*). In this method "acetone-desiccated" anterior lobe tissue is extracted repeatedly (three times) for 1 hr. at room temperature, 18–20° C., in ten volumes of 60 % alcohol at *p*H 9–10. The B fraction is separated by precipitation in absolute alcohol after adjustment of the reaction of the extract to *p*H 5. The precipitate is dehydrated in absolute alcohol and ether and finally desiccated *in vacuo* over $CaCl_2$. In three experiments the B fraction showed no virus activity (tests on four guinea-pigs). The insoluble fractions (A fractions) obtained after the extraction with 60 % alcohol at *p*H 9–10 also proved to be non-infective for guinea-pigs (four inoculated with each sample). There would appear to be little likelihood of the virus of foot-and-mouth disease surviving this method of preparation of crude whole extract of the anterior lobe tissue of the pituitary glands, especially in view of the observation recorded immediately below (see (4)). It is to be noted also that apparently the lethal effect of 60 % alcohol on the virus at 18–20° C. is increased when the medium is alkaline (Olitsky & Boëz, 1927).

(4) The effect of 60 % alcohol at room temperature, 18–20° C., on the infectivity of "acetone-desiccated" virus-infected anterior lobe tissue (pituitary gland) has been tested. Extraction with 60 % alcohol is a stage in the preparation of crude whole extract of pituitary gland by the method described above (see (3)). Virus-contaminated "acetone-desiccated" tissue became non-infective when ground in a mortar with 60 % alcohol at room temperature and left in contact for 10–15 min. Similar "acetone-desiccated" tissue was not rendered non-infective in 15 min. when treated with *absolute* alcohol under the same conditions.

(5) If any doubt existed about the possible contamination of a hormone preparation with the virus of foot-and-mouth disease, probably the most efficient way of rendering it safe would be to suspend it in a fluid, the hydrogen-ion concentration of which would then be swung at room temperature to about 4·5–5 and then back to neutrality. It would be essential to ensure that all the mixture reached the requisite *p*H and it might be advisable to maintain the *p*H at 4·5–5 for a short period—1 to 2 hr.—while thorough mixing was carried out before readjustment of the reaction was made. The question of the stability of the hormone under these conditions would have to be considered. Prolactin would presumably not be affected. Prolactin preparations were contaminated with virus. Swinging of the hydrogen-ion concentration of the mixtures at 18–20° C. to *p*H 5, then back to *p*H 7·6 was effective in inactivating the virus in six experiments (tests on four guinea-pigs). In any consideration of the possibility of the virus being rendered non-infective by usually unfavourable conditions of hydrogen-ion concentration, the protective action on the virus of high concentrations of certain salts must not be forgotten. This protective action is discussed in this paper.

(6) Pituitary glands were removed from groups of guinea-pigs infected with foot-and-mouth disease and showing generalized lesions, and tested for virus content. From the results obtained one would be justified in concluding that as far as the guinea-pig is concerned, the period of infectivity of the pituitary gland coincides with the period of infectivity of the circulating blood. The same may hold in the case of the larger animals susceptible to foot-and-mouth disease, such as cattle, sheep and pigs. The possibility of the glands becoming contaminated with other virus-infected material during the course of removal from the animal is discussed here.

(7) In the experiments described here, 15–20 g. amounts of pituitary gland were employed in each experiment. The possibility of virus surviving when large quantities of material are being dealt with under commercial conditions may be greater in the case of certain methods.

(8) The results of these experiments cannot, for reasons mentioned in the introductory remarks, be considered as absolutely decisive. However, the general conclusions that have been drawn would appear to be justifiable.

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