CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

I. Certain Basic Analyses and Observations on Lipid Components of the Virus

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Five years ago preliminary chemical analyses of elementary bodies of vaccinia, consisting of an estimation of protein, fat, and ash, were reported from this laboratory (1). The subject was not pursued further at that time because the purity of the virus preparations was a matter of some doubt. Numerous developments within the past few years have eliminated much of the uncertainty on this score. For instance, more accurate methods for obtaining infective titers of virus have been developed (2) and applied to studies on vaccinia (3); moreover, the results of experiments from two different laboratories (4, 5) seem to indicate that a single infective unit of virus is capable of initiating infection under proper conditions; and, finally, a close correlation between the number of infective units and the estimated number of elementary bodies in properly purified preparations of vaccine virus has been observed (6). Therefore, we believe that the virus preparations are now known to be of sufficient purity to warrant a thorough investigation of their various constituents. The present report deals with certain basic chemical analyses on final preparations of virus and on various fractions discarded during the process of purification. In addition, the essential nature of several lipids in the structure of the elementary body will be considered.

Materials

Preparation of Elementary Bodies of Vaccinia.—Stock suspensions of elementary bodies of vaccinia were obtained by Craigie's technique (7) from rabbits infected cutaneously with the C.L. strain of virus. This strain of virus, which was employed by Craigie in the experiments reported in 1932, has been maintained in our laboratory since 1933. During this time it has been passaged on the average of once a month by the cutaneous inoculation in rabbits of washed elementary bodies. Stock suspensions of this washed virus were highly infectious when titered intracutaneously in rabbits. Each 0.25 cc. of the 40 cc. of suspension obtained from one rabbit contained from 10^9 to 10^{10} infective units when estimated by the 50 per cent end point method (2).

In order that there be no misunderstanding about the source of the several types of material analyzed in this work, a description of the technique of purification of the virus is essential. The fur from large, healthy chinchilla rabbits with unpigmented skins was removed, by means of an electric clipper equipped with No. 0000 blades, over an area extending from the neck to the rump and down both flanks. The skin, while being held taut, was gently scraped with the edge of a folded piece of 100 mesh bronze wire clasped in a hemostat. During the scraping of each area the skin and wire were moistened with a 1:5 dilution of a stock suspension of elementary bodies. About 5 cc. of the diluted virus suspension were applied to each rabbit, and when the procedure was finished the entire shaven area, except for a narrow margin of uninoculated skin, presented a uniform pink blush. Vigorous scraping which would result in the oozing of serum or frank bleeding was avoided. On the morning of the 3rd day—and it is important that the incubation period be limited to approximately 72 hours, because if it is extended the purification of the virus is less successful—the rabbit was sacrificed by intravenous injection of air, and the inoculated area of skin was quickly removed and stretched on a board. The surface was rinsed with ethyl ether and moistened with dilute phosphate buffer solution, pH 7.2. (Buffer solution, prepared according to McIlvaine's table, was diluted 1:50 with freshly boiled distilled water and autoclaved. Solutions, pH 7.2 after autoclaving, were prepared frequently and were discarded when the reaction became neutral or slightly acid.) The moistened skin was then covered with dilute buffer solution and gently scraped with a dull knife, a total of 25 cc. of the solution being used during the procedure. The material obtained was transferred to a pyrex tube, vigorously shaken, and then spun in a horizontal centrifuge at 3000 R.P.M. for 5 minutes. Floating fatty material and hair were removed with a swab and discarded. The opalescent supernatant fluid was poured off and saved, after which 12 cc. of dilute buffer solution were added to the sediment and the tube again shaken vigorously. Centrifugation at 3000 R.P.M. for 5 minutes was again employed and the supernatant fluid was added to that obtained in the previous step. The sediment, approximately 1.0 cc. of gray pulp, was ordinarily discarded, but in certain instances was saved for comparative chemical analyses. This will be referred to as "first horizontal sediment." The pooled supernatant fluids were again centrifuged at 3000 R.P.M. for 5 minutes. The 0.1 cc. of sediment from this run, which like the first horizontal sediment was usually discarded, was saved in some instances for chemical analyses. This material will be designated as "second horizontal sediment."

The pooled fluids resulting from horizontal centrifugation, rich in elementary bodies and relatively free of large particles of debris, were next spun at 3000 R.P.M. for 1 hour in flat tubes in the Swedish angle centrifuge kept in the cold room at 0°C. This procedure sedimented the virus. After the supernatant fluid had been poured off the sediment was resuspended in dilute buffer solution. In this manner the virus was sedimented three times. The material from each rabbit was finally resuspended in 40 cc. of dilute buffer solution and spun in a 50 cc. tube on an International centrifuge at 3000 R.P.M. for 40 minutes. Recently this step also has been carried out in the cold room. Supernatant fluid from this centrifugation will be referred to as "stock suspension" of elementary bodies. The sediment which resulted from this run and which was saved for study in several instances will be designated as "third" or "final horizontal sediment." Groups of 4 rabbits were used to prepare each lot of 160 cc. of elementary body suspension.

Lots of virus were titered within a few days after preparation by intracutaneous inoculation into rabbits of the Havana breed. During the past year, 2 areas were inoculated respectively with 0.25 cc. of the 10^{-7} and 10^{-8} dilutions of each lot of virus, while 8 areas were inoculated with the 10^{-9} and 10^{-10} dilutions, respectively. Various solutions have been employed for diluting the virus for titration, namely, dilute phosphate buffer solution, Locke's solution which contained 5 per cent fresh inactivated normal rabbit serum, a mixture of 60 per cent dilute phosphate buffer and 40 per cent Locke's solution to which was added 5 per cent rabbit serum, and, finally, a mixture of equal parts of 10 per cent dextrose solution and dilute phosphate buffer to which was added 5 per cent normal serum. The last 2 mixtures appeared to give the most consistent results.

Lots of virus suspension, with a few cc. of anhydrous ethyl ether added to inhibit growth of bacteria, were stored at 3°C. until 5 to 8 of them were collected; this usually required several weeks. The lots of elementary bodies were pooled, concentrated in the ultracentrifuge, and washed with dilute phosphate buffer solutions first at pH 6.0, and then at pH 8.0, and, finally, with several changes of distilled water, following which the virus was dried from the frozen state (6). After further dehydration over phosphorus pentoxide the preparations were weighed. The procedure for estimating the number of elementary bodies in a given pool from the dry weight of the material as well as the method of obtaining the infective unit-elementary body ratio of each preparation has been described in a recent paper (6) in which appeared data regarding the infectivity of 5 of the 11 pooled preparations analyzed in the work described in the present paper.

Methods of Chemical Assay

Because the microchemical methods employed in these analyses may serve in some instances to explain certain discrepancies between data to be presented here and those reported elsewhere (1, 8, 9), the procedures used, particularly those for fat fractionation studies, will be described in some detail.

Lipid Analyses.—Estimation of lipid by weighing alcohol-ether extractable material is subject to errors of great magnitude. For example, Folch and Van Slyke (10) and Christensen (11) have recently called attention to the occurrence of protein, amino acids, chlorides, phosphates, and urea in alcohol-ether extracts; these non-lipid materials were shown to account for as much as 80 per cent of the "fatty" material in some biological preparations. Methods of lipid analysis developed by Kirk, Page, and Van Slyke (12) and modified by Folch and Van Slyke (13) were employed throughout our work. In this method of fat fractionation due cognizance has been taken of non-lipid materials which contaminate alcohol-ether extracted lipids; furthermore, manometric methods afforded an analytical precision in the examination of the relatively small amounts of materials available which was not obtainable by other techniques. In our hands, these methods have shown that in some instances as much as 50 per cent of the alcohol-ether extractable substances of dried elementary bodies may be non-lipid in nature. Errors of this type were obviated by the use of non-polar solvents for re-extraction of the evaporated residue of the alcohol-ether extracts.

A representative lipid determination which serves to illustrate the methods employed is presented in the following protocol.

20 mg. of purified elementary body preparation, dried to constant weight, were extracted for 2 hours with 15 cc. of a boiling, 3:1 alcohol-ether mixture in a 25 cc. flask with a condenser of the Graham variety. Longer periods did not increase the yield of lipid, but increased considerably the amount of non-lipid substances. After cooling to room temperature, the mixture was filtered rapidly through a fat-free Seitz pad, and made up to 25 cc. in a volumetric flask with several successive portions of alcohol-ether mixture drawn through the filter pad. That no fat was lost in this manner was shown by the inability to recover lipid material from several discarded Seitz pads. 20 cc. were transferred to a beaker, and evaporated to dry residue on a water bath at 85°C. under partial vacuum. Reextraction of dry residue was carried out with several small portions of warm redistilled petrol ether which were then pooled and transferred to a sintered glass funnel and filtered into a 20 cc. volumetric flask. In this manner the original ratio of weight of elementary body preparation to volume of extraction fluid was maintained throughout successive fractionation. 2 cc. aliquots of the petrol ether extract were pipetted into combustion tubes, evaporated to dryness in the water bath, and combusted to carbon dioxide which was measured manometrically according to the method of Kirk, Page, and Van Slyke (12).

Total Lipid.—The total lipid values were calculated as mixed lipids.

Cholesterol.—1 cc. aliquots of the alcohol-ether extract were pipetted into combustion tubes and the cholesterol was precipitated as the digitonide. The precipitate was caught on a filter stick and combusted in the manner described for total lipid. The cholesterol was calculated from the carbon dioxide given by combustion of the cholesterol-digitonide complex.

Cholesterol Esters.—No increase in cholesterol was obtained after saponification, hence it was concluded that the cholesterol was present entirely as free, or non-esterified cholesterol.

Phospholipid.—Phospholipid was determined from the lipid phosphorus in the petrol ether extract. The phosphorus analyses were performed on a digest of the petrol ether residue, and the amount was determined by combustion of the strychnine phosphomolybdate precipitate, according to the method of Kirk, Page, and Van Slyke (12). The phosphorus-nitrogen ratio of the petrol ether residue was approximately 1:1, indicating that the phospholipid was lecithin.

Neutral Fat.—Neutral fat was estimated from the value for total lipid by subtracting the sum of the values for cholesterol and phospholipid.

Nitrogen.—Nitrogen was estimated by the Van Slyke procedure (14) following digestion with sulfuric acid and selenium oxychloride.

Amino Acids.—Alpha-amino acids were determined, following hydrolysis by 6 N hydrochloric acid, by the ninhydrin alpha-amino carboxyl technique of MacFadyen and Van Slyke (15). This method was particularly suitable for the small amounts of material available. 5 mg. of elementary bodies were sufficient for an accurate determination.

Cystine.—Cystine was estimated by the nitroprusside reaction (16). There was no inorganic sulfur, as shown by the benzidine precipitation method (17).

Reducing Sugars.—These were estimated by the method of Shaffer and Somogyi (18) following hydrolysis with 2 N hydrochloric acid.

Organic Phosphorus.—Organic phosphorus was determined by the method of Kirk

(19) on samples of dried virus prepared according to routine. Tests for inorganic phosphorus were negative on final preparations of elementary bodies. A correction was applied to the data obtained on horizontal sediments which contained appreciable amounts of inorganic phosphorus.

EXPERIMENTAL

Analyses of Final Preparations of Elementary Bodies

Dried material from eleven preparations of elementary bodies of vaccinia was subjected to chemical analysis. The purity of the different lots was of

TABLE I

Analyses of Elementary Bodies of Vaccinia

Lot	IU:EB ratio*	Phosphorus	Total nitrogen	Alpha-amino N after hydrolysis	Carbon	Lipid				Cystine after hydrolysis	Reducing sugars after hydrolysis†
						Total lipid	Cholesterol	Phospholipid	Neutral fat		
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
46	1:7.6	0.59	15.3	3.6	34.2	5.1	1.1	2.1	2.0		3.0
47	1:8.8	0.59	15.6	3.1	34.9	6.1	2.2	1.8	2.1	1.8	2.3
48	1:2.7	0.50	15.1	2.5	33.6	5.0	1.1	2.0	1.9	1.6	3.2
53	1:4.7	0.58	15.6	3.7	32.6	5.0	1.0	2.2	1.8		2.0
55	1:3.0	0.49	15.3	3.7	31.8	4.3	1.0	1.7	1.6	2.0	2.6
56	1:3.7	0.59	14.8	2.8	34.5	6.1	1.2	2.5	2.4		3.5
57	1:9.2	0.58	15.2	3.1	33.8	5.3	1.1	2.2	2.0		3.0
58	Not done	0.57	15.2	3.5	34.8	4.9	1.0	1.9	2.0		3.1
66	1:4.2	0.59	14.9	3.6	34.6	8.1	2.1	2.8	3.8		2.5
69	1:3.7	0.62	15.4	3.2	32.5	6.5	1.8	2.1	2.6	1.8	2.3
70	1:1.5	0.57	15.5	4.1	34.2	6.2	2.0	2.5	1.7	2.3	3.5
Average...	1:4.9	0.57	15.3	3.4	33.7	5.7	1.4	2.2	2.2	1.9	2.8

Values of all chemical analyses are expressed as per cent of dry weight.

* IU:EB ratio = infective unit-elementary body ratio.

† Expressed as glucose.

a relatively high order, since the infective unit-elementary body ratios varied between 1:1.5 and 1:9.2, averaging 1:4.9. Values for total nitrogen, alpha-amino nitrogen, carbon, carbohydrate, cystine, total lipid, cholesterol, phospholipid, and neutral fat were obtained and expressed as percentage of dry weight of the virus. The data are summarized in Table I.

The summarized data in Table I show a striking consistency. This is particularly evident in the analyses, *e.g.*, phosphorus, nitrogen, and carbon, which can be done directly and with considerable accuracy on the small amounts of material with which it was necessary to work. Virus lots 46 and 47, both of which contained 0.59 per cent phosphorus, had been previously analyzed for this element (9) by a different technique with resultant

values of 0.44 and 0.43 respectively. The phosphorus-nitrogen ratios of the phospholipid were approximately 1:1.

TABLE II

Successive Analyses on "Horizontal Sediments" Discarded during Preparation of Elementary Bodies

Sample from rabbit No.	Phosphorus	Total nitrogen	Alpha-amino N after hydrolysis	Carbon	Lipid				Cystine after hydrolysis	Reducing sugars after hydrolysis*
					Total lipid	Cholesterol	Phospholipid	Neutral fat		
	"First Horizontal Sediments"									
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	1.01	11.1	2.1	35.1	11.2	2.1	1.6	7.5	4.5	1.1
2	1.06	12.8	2.2	36.8	12.1	1.9	1.2	9.0	3.9	2.2
3	1.00	12.2	2.7	36.9	11.6	2.2	1.8	7.2	4.2	1.1
4	1.01	11.7	1.7	34.2	9.8	1.6	2.0	6.2	3.9	2.3
Average.....	1.02	12.0	2.2	35.8	11.2	2.0	1.7	7.5	4.1	1.7
	"Second Horizontal Sediments"									
1	0.91	13.7	1.9	34.5	8.2	1.8	1.2	5.2	1.6	1.1
2	0.81	13.8	2.0	34.8	7.6	1.6	1.2	5.2	1.2	2.1
3	0.72	12.1	2.4	36.2	9.7	1.8	1.5	6.4	1.0	1.1
4	0.61	12.2	1.4	36.2	7.9	1.5	1.8	4.6	1.6	1.2
Average.....	0.76	13.0	1.9	35.4	8.4	1.7	1.4	5.4	1.4	1.4
	"Third (Final) Horizontal Sediments"									
1	0.61	12.8	1.9	32.6	7.1	1.8	1.2	4.2	1.2	1.2
2	0.59	13.5	1.8	31.8	7.0	1.2	1.2	4.6	1.5	2.1
3	0.70	13.0	1.7	34.2	8.0	1.9	2.0	4.1	1.2	1.4
4	0.68	13.9	1.9	33.1	6.9	1.2	1.7	4.0	1.6	2.3
Average.....	0.65	13.3	1.8	32.9	7.2	1.5	1.5	4.2	1.4	1.8
	Final Preparation of Elementary Bodies Separated from Above Sediments									
	0.57	15.5	4.1	34.2	6.2	2.0	2.5	1.7	2.3	3.5

Values of all chemical analyses are expressed as per cent of dry weight.

* Expressed as glucose.

Chemical Nature of Material Discarded during Process of Purification of Elementary Bodies

In Table II are presented separate analytical data on three fractions of dermal pulp which were collected from 4 infected rabbits and which would ordinarily have been discarded during the process of purification of the virus; in addition, values on the final preparations of elementary bodies

obtained from these 4 animals are included. In order to avoid introducing inorganic phosphorus present in the ordinary buffer solution, the dermal pulp was suspended and washed with 0.01 molar lithium-veronal buffer solution, pH 7.9. It is at once apparent that the percentages of total and carboxyl nitrogen, and of reducing sugars progressively increased as the final or purified preparation of elementary bodies was approached. On the other hand, the percentages of organic phosphorus and of total and neutral fat progressively decreased in successive fractions that were separated, and were lowest in the final virus preparation. Cystine, which was present in large amounts in the "first horizontal sediment," was reduced in the "second and third horizontal sediments," but was again present in increased amounts in the final elementary body preparation.

In general, one can say that significant differences were noted in the amounts of phosphorus, total nitrogen, alpha-amino nitrogen, total lipid, neutral fat, reducing sugar, and cystine in the various types of materials discarded during the purification of the virus and in the amounts of these constituents in the final or purified preparation of elementary bodies. Furthermore, variations in the determinations on different lots of the same types of material stood out in contrast to the constancy observed in the values obtained in the final elementary body preparations.

The Rôle of Lipids in Elementary Bodies

The progressive diminution in the total lipids in the various fractions that were ordinarily discarded during the process of obtaining purified elementary bodies, strongly suggests that a value much in excess of 6.0 per cent for the final virus preparation indicates a contamination with non-essential fatty material. On the other hand, the failure to obtain a reduction in the lipid content of purified elementary bodies below 4.3 per cent is perhaps some evidence for assuming that this amount of fat may be an integral part of the virus. Two lines of investigation were followed to determine whether one or more of the lipid fractions obtained from elementary body preparations are integral constituents of the virus.

Extraction of Virus with Ethyl Ether.—Ethyl ether has long been known to have little or no inactivating effect on vaccine virus. In fact, stock suspensions of elementary bodies in buffer solution are saturated with ether to prevent bacterial growth during storage (7). Moreover, McFarlane and associates (8) have shown that extraction of dried virus with ethyl ether at room temperature does not reduce appreciably the infectivity of the material. We have also found no inactivation of the virus when it is extracted

with ethyl ether in the cold as evidenced by the results of the following experiment.

Elementary body pool No. 69, whose infective unit-elementary body ratio was 1:3.7, contained 407.0 mg. of dry material. 100 mg. were employed for chemical analyses, the results of which are presented in Table I. 301.7 mg., available for this experiment, had been stored under vacuum over phosphorus pentoxide at 0°C. during an interval of 6 weeks after having been dried from the frozen state. 12.5 mg. were removed for titrations of infectivity and stored under the same conditions; the remaining portion, 289.2 mg., was suspended in 50 cc. of cold anhydrous absolute ethyl ether and stored in a stoppered container at 0°C. for 48 hours; the suspension was thoroughly shaken on a number of occasions. The virus was sedimented from the ether by centrifugation in the angle centrifuge in the cold and again suspended in 50 cc. of ether. This extraction

TABLE III

Analyses of Elementary Bodies before and after Extraction with Ethyl Ether

	Elementary bodies before extraction	Elementary bodies after extraction	Ether extract residue
	per cent	*per cent*	*per cent*
Total lipid	6.5	5.0	1.62
Total nitrogen	15.4	15.6	0.00*
Cholesterol	1.8	0.00*	1.6
Phospholipid	2.1	2.2	0.00*
Neutral fat	2.6	2.8	±0.1

Values are expressed as per cent of dry weight of virus.

No appreciable loss of infectivity after extraction.

* Trace, insufficient quantity for estimation.

likewise proceeded for 48 hours at 0°C. when the suspension was again centrifuged. The supernatant ether was added to the first extract and saved. The ether extracted virus was dried under vacuum and weighed; 278.0 mg. were recovered. Hence, the material lost during extraction amounted to 11.2 mg. or 3.9 per cent of the original preparation. Results of analyses on the ethereal extract and on the treated virus are presented in Table III; for convenience the results obtained before extraction are repeated from Table I.

15 mg. of ether extracted virus were resuspended in 30 cc. of a dilute buffer solution containing 5 per cent of fresh normal inactivated rabbit serum, the final pH of the mixture being 7.7 (glass electrode). The method recommended by McFarlane and associates (8) was employed in resuspending the elementary bodies; this consisted of breaking up the aggregated clumps of elementary bodies in a TenBroeck grinder (20), an illustration of which was reproduced in their article. Instead of grinding entirely by hand, however, it was found expedient to insert the top of the pestle into a rubber stopper placed on the shaft of an ordinary stirring machine and to complete the process by mechanical power. In a similar manner, the 12.5 mg. of dry unextracted virus were suspended in 25 cc. of fluid. The method of resuspension was quite efficient, since the

majority of particles appeared to be monodispersed when smears stained by Morosow's technique (21) were examined. Moreover, each of the aggregates that remained was composed of only a few elementary bodies. Three rabbits were each inoculated intracutaneously with dilutions of both preparations and the results are summarized in Table IV.

The data summarized in Table IV show that no appreciable inactivation of virus occurred as the result of ether extraction. Both the dry virus and

TABLE IV

Titration of Resuspended Dry Elementary Bodies before and after Extraction with Ethyl Ether

Preparation	Log dilution	No. of inoculation	No. positive	No. negative	Accumulative positive	Accumulative negative	Per cent positive	Per cent negative	Infective units per ¼ cc. of suspension
							per cent	*per cent*	
Unextracted	−5	3	3	0	40	0	100	0	$10^{8.6}$, or 3.9 $\times 10^8$
	−6	6	6	0	37	0	100	0	
	−7	10	10	0	31	0	100	0	
	−8	18	14	4	21	4	84	16	
	−9	18	6	12	7	16	30	70	
	−10	12	1	11	1	27	4	96	
Extracted	−5	3	3	0	38	0	100	0	$10^{8.5}$, or 3.2 $\times 10^8$
	−6	6	6	0	35	0	100	0	
	−7	10	10	0	29	0	100	0	
	−8	18	12	6	19	6	76	24	
	−9	18	6	12	7	18	28	72	
	−10	12	1	11	1	29	3	97	

Infective unit-elementary body ratio of original material based on titration before drying was 1:3.7. Both resuspended preparations contained 0.5 mg. of dry material per cc. Therefore, on the basis of their infective titers, the IU:EB ratios were 1:241 and 1:294 for the unextracted and extracted portions, respectively.

the dry ether-extracted virus were resuspended in sufficient fluid to give a concentration of 0.5 mg. per cc. Therefore, since the infective units per 0.25 cc. were $10^{8.6}$ for the former and $10^{8.5}$ for the latter, their infective unit-elementary body ratios were 1:241 and 1:294, respectively. Sprunt and McDearman (5) have found that titration end points of vaccine virus suspensions obtained by this method are reproducible within 0.08 of a log dilution; the method is about as satisfactory in our hands. Although ether extraction did not affect the activity of the dry virus, it is to be noted that drying, storage, and resuspension result in an appreciable inactivation.

The analytical results presented in Table III indicate that at least one of the lipids, namely, cholesterol, is apparently not essential to the virus,

since it was removed almost completely without appreciably affecting the infective titer of the preparation. Saponification has not been found to increase the digitonide precipitating substance of the virus, hence it is likely that cholesterol is present only in the unesterified form. It seems reasonable to believe that cholesterol is merely adsorbed on the virus; this agrees with McFarlane's ideas (8).

Neutral fat and phospholipid were not appreciably altered by extraction with anhydrous ethyl ether in the cold, Table III. This would at least suggest that the neutral fat was not merely adsorbed on the virus particle; however, the essential nature of these lipids in the elementary body cannot be assessed from this experiment. Inasmuch as their removal from wet elementary bodies by extraction with alcohol and ether in the cold (22) or their removal from dry elementary body preparations by a mixture of these solvents (8) in each case results in inactivation of the virus, it might possibly be assumed that these constituents are essential to the integrity of the virus. Such a conclusion is not justified, however, because, as has already been pointed out (8), alcohol may inactivate the virus by some mechanism other than extraction of the lipid.

Action of Pancreatic Lipase on Vaccine Virus.—The effect of pancreatic lipase on the virus of vaccinia and on the lipids present in preparations of the virus was investigated in the following manner.

A water soluble globulin, possessing marked lipase activity when tested on tributyrin and triolein substrates, was prepared from a sodium chloride extract of acetone extracted pancreatin by repeated ammonium sulfate precipitation (23). 1 cc. of a water-clear solution of this material was sufficient to hydrolyze 0.05 gm. of tributyrin in 1 hour. 2 cc. of the solution were incubated for 1 hour with 50 mg. of vaccine virus, preparation No. 66, suspended in dilute phosphate buffer, 1 per cent sodium taurocholate being added to activate the lipase. At the end of the hour the virus was subjected to ultracentrifugation, and washed thoroughly in distilled water with repeated centrifugation. A portion was reserved for a study of infective titer, while the remainder, after being dried in the frozen state to constant weight, was subjected to chemical analysis. The results are summarized in Table V.

From the data in Table V it is at once apparent that pancreatic lipase, which had previously been shown to be active in the rapid hydrolysis of triolein and tributyrin, did not alter significantly the fat content of the virus preparation. The value for the percentage of neutral fat, determined before and after treatment on the basis of dry weight of virus, was, within the limits of the method, essentially unchanged. Moreover, the infectivity of the virus was not significantly altered by the action of lipase as shown by tests after treatment with active and heat-denatured enzyme; in one such

experiment the titer of both suspensions was $10^{-8.7}$. The latter finding is in accord with an observation made by Pirie (23) who found that the alleged inactivation of vaccine virus by lipase preparations was not due to a lipolytic action but to the presence of free fatty acids and lecithin in the material used as a source of lipase.

Two hypotheses immediately present themselves. In the first place, the fat might be bound in some manner whereby lipase is incapable of effecting hydrolysis. Such might be the case if the fat were built into a lipoprotein complex, or if it were stored within a membrane-like structure which

TABLE V

Action of Pancreatic Lipase on Elementary Bodies

Preparation	Total lipid	Cholesterol	Phospholipid	Neutral fat
	per cent	*per cent*	*per cent*	*per cent*
Before lipase treatment	8.1	2.1	2.8	3.8
After lipase treatment	8.0	1.9	2.6	3.5

Values are expressed as per cent of dry weight of virus.

TABLE VI

Hydrolysis by Pancreatic Lipase of Fat Extracted from Elementary Bodies

Amount of virus taken for experiment, *mg*	20.0
Amount of neutral fat extracted (3.8 per cent of virus), *mg*	0.76
Amount of fat recovered by wet extraction after lipase, *mg*	0.11
Amount of neutral fat hydrolyzed by lipase, *mg*	0.65
Neutral fat hydrolyzed in 4 hours, *per cent*	86.0

did not admit the lipase molecule. Secondly, the material represented as neutral fat might be peculiarly constructed and resistant to lipase hydrolysis. Such fats are known and have formed the basis of a recent study by Kelsey (24).

In order to test the second hypothesis, the following experiment was performed.

The petrol ether extract from 20 mg. of elementary bodies (preparation No. 66) was emulsified mechanically with 1 cc. of the lipase preparation described above and incubated at 38°C. Sodium taurocholate, sufficient for a final concentration of 0.01 per cent, was added for activation. A blank determination consisting of 1 cc. of lipase and the same amount of sodium taurocholate was set up and likewise incubated. After 4 hours the solutions were titrated with 0.005 N carbonate-free sodium alcoholate to a phenolphthalein end point. The material was next extracted in the wet with alcohol and ethyl ether, and the total unhydrolyzed lipid determined by combustion of the petrol ether soluble portion of the extract.

It is clear from the results summarized in Table VI that the neutral fat in vaccine virus is not unusual as regards its susceptibility to hydrolysis by pancreatic lipase. It would seem that in the intact elementary bodies the physical arrangement of the neutral fat is such as to render it incapable of enzymic hydrolysis.

DISCUSSION

The average values obtained in the chemical analyses of eleven lots of dry washed elementary bodies of vaccinia were the following: total nitrogen, 15.3 per cent; alpha-amino nitrogen after hydrolysis, 3.4 per cent; total carbon, 33.7 per cent; total phosphorus, 0.57 per cent; cholesterol, 1.4 per cent; phospholipid (lecithin), 2.2 per cent; neutral fat, 2.2 per cent; reducing sugars after hydrolysis, 2.8 per cent; cystine, 1.9 per cent.

A nitrogen value of 15.3 per cent is somewhat higher than heretofore reported for washed elementary bodies (1, 8, 25). The total amount of phosphorus is likewise slightly greater than that recently reported from this laboratory (9). The rise in our present figure for the amount of phosphorus is not, like the rise in the nitrogen value, dependent on increased purity of the virus preparations, but is to be explained on the basis of a more accurate microchemical technique. The present values for nitrogen and phosphorus in dried washed elementary bodies approach closely those obtained by McFarlane and associates (8) on their virus preparations after extraction with alcohol and ether. Our figures for the lipid content of dried washed elementary bodies are definitely lower than the ones recorded by the British workers. These discrepancies are due in part to methods used and in part to materials analyzed. As to the latter, it has been clearly demonstrated that the total lipid value of each successive "horizontal sediment" obtained during the process of purification falls progressively and is lowest in the final or purified virus preparations. Therefore, one is led to believe that total lipid and phospholipid values greater than 6.0 and 2.2 per cent, respectively, are indications of contamination by materials from crude pulp.

The present observations strongly suggest, but do not prove, that cholesterol is not an essential constituent of the elementary body, since it was readily removed by ether extraction without appreciably affecting the infectivity of the virus. On the other hand, neutral fat, which in the free state was soluble in ether and hydrolyzed by lipase, was not reduced in elementary bodies by treatment with these agents—agents that do not impair the infectivity of the virus. In addition, the amount of phospholipid in elementary bodies was not diminished by treatment with these agents. It remains to be shown whether neutral fat and phospholipid are

integral components of the virus particle; nevertheless, the evidence is consistent with the idea that they are important parts of its structure.

Phosphorus in the phospholipid constituent of elementary bodies represents only about 0.08 per cent of the total weight of the virus. Previous observations (8, 9) lead us to believe that the major portion of the non-lipid phosphorus, approximately 0.5 per cent of the virus, is to be found in the thymonucleic acid fraction of the active agent. Application of the usual conversion factor to this amount of non-lipid phosphorus shows that 5.0 per cent of the elementary body is thymonucleic acid. The content of carbohydrate in this amount of nucleic acid would be sufficient to account for most of the reducing sugars detected after hydrolysis of the elementary bodies. That part of the reducing sugar may be derived from sources other than desoxyribose is to be expected since small amounts of glucosamine have been found (8, 9) and since the heat stable soluble antigen of vaccinia, which can be extracted from dried virus (9), possibly contains some carbohydrate.

The relative consistency of the carbon values in the successive discarded materials and in the final virus preparations is not surprising. Amounts of cystine in the various fractions appear to show a trend different from those of the other constituents studied. As one would expect, this substance constitutes an appreciable amount, 4.1 per cent, of dermal pulp, since keratin contains as much as 18 per cent cystine; then the amount diminishes in the next two "horizontal sediments;" and finally it rises to about 2.0 per cent in the purified material.

SUMMARY

The results of chemical analyses reveal that it is possible to secure preparations of elementary bodies of vaccinia that possess a considerable uniformity in their chemical constituents. Furthermore, the amounts of certain of these constituents, namely, nitrogen, alpha-amino nitrogen after hydrolysis, phosphorus, total fat, phospholipid, neutral fat, reducing sugar after hydrolysis, and cystine, in the purified virus are significantly different from those in the various materials discarded during the process of purification.

The amounts of phospholipid and neutral fat in the virus preparations are not appreciably affected by extraction with ethyl ether or by digestion with lipase, procedures which do not inactivate the virus. Cholesterol, on the contrary, is apparently completely removed by these manipulations, and hence is not considered to play an important part in the economy of the virus.

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