Biochemical Changes and Incorporation of P^{32} in Separated Elements of the Hyperplastic Lesion of Fowlpox. (26197)

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Investigation of an animal virus in the medium of the intact host has been superseded in some instances by the more quantitative approach of tissue culture technic utilizing a well-defined cell type. Where feasible, the advantages of tissue culture are conceded, but in the case of fowlpox the virus has not yet been cultivated in an established cell line. The nature of the lesion necessitates investigation in animals since the classical hyperplasia and inclusion body formation are scarcely evident in tissue culture.

It is well known that fowlpox infection involves the skin with conspicuous hyperplasia of the epithelium and thickening of the connective tissue due to hyperplasia and associated inflammatory changes. Concomitant with these events the large conspicuous intracytoplasmic inclusions develop in the epithelial cells. Because of the amount of lipid evident in the inclusion body an investigation of lipid content of whole chick skin and chorioallantoic membrane was undertaken(1). It appeared likely that analysis of whole tissue would not properly portray differences that might occur in the several layers of the skin such as the epithelium which is not invaded by inflammatory cells. Methods were devised to separate the skin into epithelial elements and connective tissue. The present experiment describes differences in the biochemistry and incorporation of P^{32} in the separated structures of fowlpox infected skin.

Materials and methods. Virus. The strain of fowlpox and preparation of inoculum used in this study have been documented(1).

Experimental animals and separation of tissue. Day-old cockerels obtained from a local hatchery were inoculated by gently rubbing a 1:5 dilution of stock material on the freshly plucked scalp, resulting in a confluent lesion. After the desired interval of infection, chicks were dispatched with ether and the scalps immediately excised, carefully rinsed in saline, drained, and placed in 1% Bacto trypsin in 0.02 Tris buffer (pH 7.6) for 17 to 20 hours at 4°C. The tissue then was gently rinsed free of trypsin solution with 0.9% NaCl and allowed to drain. The superficial epithelium could now be easily removed, with curved forceps, as a soft paste, and the turgid, greatly enlarged, hyperplastic follicles expressed from the supporting connective tissue by gentle pressure. After they had been rinsed thoroughly in physiological saline to remove debris, all tissues were frozen and lyophilized to constant weight.

Chemical procedures. The dried tissues were extracted exhaustively at room temperature with chloroform-methanol (2:1). The extracts were taken to dryness under nitrogen (N), and the lipids were repeatedly extracted from the residue with petroleum ether (B.P. 30-60°C). After evaporation under N total lipids were dried to constant weight and redissolved in a known volume of petroleum ether for isotopic and chemical analysis. The methods for performing the various chemical procedures have been noted previously(1), and only the lipid fractions that showed statistical variation as the result of infection were employed in the present study. The possibility that the extracted phospholipid was contaminated with highly radioactive inorganic P^{32} was investigated. Exhaustive experiments in this laboratory reveal that no detectable inorganic P^{32} can be carried over into petroleum ether extracts.

Isotopic procedures. The experiment utilized inorganic P^{32} as $H_3P^{32}O_4$ in HCl solution obtained from Oak Ridge National Laboratory. Quantitative dilutions were made with 0.9% NaCl containing 5 mg of P as

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TABLE I. Lipid Composition of Separated Components of Normal and Infected Chick Skin.*

Tissue	Total lipid†		Phospholipid		Total cholesterol		Free cholesterol		Esterified cholesterol	
	Norm.	Inf.	Norm.	Inf.	Norm.	Inf.	Norm.	Inf.	Norm.	Inf.
Epithelium										
3 days	39.6	38.8	7.8	14.2	4.0	4.8	N.S.	3.3	N.S.	1.5
5 "	38.8	35.6	6.2	15.7	3.6	8.6	N.S.	4.2	N.S.	4.4
7 "	40.4	33.5	6.3	15.8	3.1	13.5	1.6	5.3	1.5	8.2
Follicles										
7 days		29.3		22.6		17.1		5.9		11.2
Connective tissue										
3 days	29.0	23.3	19.7	26.7	12.2	16.1	6.1	8.8	6.0	7.3
5 "	32.0	14.5	15.9	38.7	9.2	24.1	4.8	14.8	4.4	9.4
7 "	30.7	17.6	17.2	46.4	9.0	18.8	4.4	13.1	4.7	5.7

* Lipid fractions expressed as % of total extractable lipid.
† Total lipid expressed as % of dry tissue wt.
N.S. = Not sufficient material for analysis.

$PO_4(H_3PO_4)$ per liter, as recommended by Zilversmit(2).

Each chick was given approximately 15 μc of P^{32} intramuscularly, and samples of appropriate dilutions of the inoculum were evaporated to dryness on nickel-plated, one-inch diameter planchets, the bottoms of which were lined with 3-mm Whatman paper. (The paper assured uniform distribution of the lipid samples which were to be counted under the same conditions.) The inoculum planchets served as a check on the day-to-day variations in counting efficiency as well as a measure of the isotope given each chick. Activity was determined with a Tracerlab End-Window Geiger Tube and Superscaler. Counts were made to a probable error of $\pm$ 1% and corrected for decay according to the widely used expression $A_o = A/e^{-kt}$ Lipid samples were counted in triplicate in amounts less than 50 mg per planchet. It is pertinent that this amount of inactive lipid could be added to a previously counted inoculum planchet without evident loss of activity due to absorption(3). As the result of preliminary uptake studies it had been determined that maximum incorporation of P^{32} in the skin of the chick occurred 48 hours after administration and that administration of isotope caused no overt changes in lipid composition of the tissues under study. Accordingly, in subsequent experiments all chicks were harvested 48 hours after injection of isotope. For each of the several trials 90 infected and 90 controls received isotope 48 hours before sacrifice. Thirty chicks in each group were killed at 3, 5, and 7 days.

The 3-day period was arbitrarily chosen as the starting point since the skin at this time shows the earliest evidence of infection, and subsequently the lesion develops rather rapidly. The various periods here refer to duration of the experiment and not to age of the chick which was, on the average, 24 hours old at the onset.

Results. Lipid changes. It has already been stated that P^{32} administration did not produce demonstrable isotopic effect on the lipid chemistry of intact normal chick skin.

The lipid changes during course of infection are better compared with the normal when the skin is separated into connective tissue and epithelial elements, *i.e.,* surface epithelium and follicles. These differences are depicted in Table I and represent the average of triplicate analysis of 2 different trials for each time interval. It should be noted that as a group there is a distinct difference between normal epithelium and connective tissue in each of the categories. A comparison of infected epithelium and connective tissue with its normal counterpart shows approximately a 2- to 3-fold or greater increase in phospholipid and total, free, and esterified cholesterol at some time during infection.

It should be noted that during infection one of the components (epithelial phospholipid) did not increase, having reached the

TABLE II. Specific Activities of Separated Layers of Normal and Infected Tissues.*

	Epithelium		Connective tissue		
Hr†	Norm.	Inf.	Norm.	Inf.	Follicles
72	.74	.66	.56	.64	
72	.71	.77	.56	.67	
120	.36	.59	.35	.58	
120	.33	.64	.33	.55	
168	.30	.57	.27	.47	.48
168	.32	.53	.25	.47	.48

* Specific activity $= \frac{\text{CPM/mg P}}{\text{CPM/chick}} \times 100 = \frac{\%\text{ inj. } P^{32}}{\text{mg phospholipid P}}$.

† Hr represent time of sacrifice of chicks and, where pertinent, post-inoculation of virus. Each group received isotope 48 hr before death.

maximum value earlier in development of the lesion. The data concerning the follicles require some comment. The study of these structures is limited to the 7-day period since it is difficult to obtain them in any quantity before this time. Furthermore, comparison with the normal follicle is not possible since these structures cannot be separated from the surrounding tissue. Since the infected follicle is largely composed of hyperplastic epithelium, one might expect the lipid categories to be similar to those of the surface epithelium. With the exception of free cholesterol all of the values are appreciably higher. Finally, for all categories the sum of the components does not approximate 100%. The difference is probably due to free and total fatty acids(1).

Effect of infection on incorporation of P^{32}. A comparison of specific activities (SA) of phospholipid P in the several epithelial structures and connective tissue at different time intervals is shown in Table II. A comparison of SA of normal epithelium and connective tissue at 72 hours reveals that incorporation of P^{32} is about 20% less in the latter. In the older chicks SA of both normal tissues drops sharply, approximately 50% in 7 days. Average specific activity of infected tissues at 72 hours is approximately equal to that of the normal counterpart. However, at 5 and 7 days average incorporation drops slightly and consistently in connective tissue and to a lesser degree in surface epithelium, and in any case is approximately 1.5 to 1.7 times that of the normal controls. It is somewhat surprising that surface epithelium and follicular epithelium have different specific activities. Follicular epithelium and connective tissue exhibit the same activity.

Discussion. It has previously been remarked that administration of P^{32} did not result in a demonstrable isotopic effect so far as the lipid components determined here are concerned. Although the significance is not apparent it is worth noting that the phospholipid content did not change appreciably in noninfected whole skin or in the separated elements, whereas specific activity of the phospholipid phosphorus of normal epithelium and connective tissue was reduced by approximately 50% at 5 and 7 days.

It has been shown here that, as a result of infection, cholesterol components and phospholipid increased percentage-wise in both epithelium and connective tissue, though total lipid of the epithelium did not vary significantly and was considerably decreased in the connective tissue (Table I). The refined data did not include weight of the tissue from which the lipid was extracted. Concerning this relation, the ratio of tissue weight/lipid weight averaged 2.5 for normal epithelium and 2.9 for infected, at 5 and 7 days. On the other hand, weight of infected connective tissue increased considerably over amount of lipid as ratios of normal to infected changed from 3.2 to 6.3. Other than hyperplasia as a contributing cause of added weight of connective tissue, consideration must be given to the contribution made by inflammatory cells which are quite abundant. Of course, a class of compound other than lipid could contribute materially to the added weight.

The apparent increase in lipid phosphorylation reported here as a result of virus infection is, of course, not new. Miroff *et al.*(4) demonstrated a significant increase in P^{32} incorporation into the phospholipids of HeLa cells infected with poliovirus. Moldave(5) found that mouse brain phospholipid has a higher specific activity as a consequence of infection with Theiler's GD VII virus, 1.3 times that of the controls. Infection of KB cells with adenovirus also stimulated uptake of P^{32} into lipid fractions(6). There appears

to be no explanation for the increase or disposition of phospholipid molecules during infection. The matter is further complicated by reason of extensive hyperplasia concomitant with virus replication. In this regard Levin(7) cited previous experiments indicating that phospholipid metabolism is stimulated during certain conditions associated with hyperplasia.

The decreasing specific activity noted in noninfected epithelium and connective tissue might be due either to lack of uptake or to an increased turnover rate. On the other hand, the elevated specific activity (gradually decreasing with time) of infected tissues could be due to an increased synthesis of phospholipid compounds or a normal rate of synthesis with deposition in areas of compounds where there is little turnover.

Summary. Normal control and fowlpox-infected chick scalp were separated by trypsin into connective tissue and epithelium. By appropriate technics the infected epithelial tissue was separated into surface epithelium and follicles. Comparison was made between normal epithelium and connective tissue and between normal and infected tissues with respect to certain lipids and incorporation of P^{32} into phospholipid. Distinct differences in phospholipid and total, free, and esterified cholesterol were found between normal epithelium and connective tissue. The above components were increased 2- to 3-fold at some interval of time in both infected epithelium and connective tissue. The specific activities of phospholipid P from all tissues were approximate at 3 days but that of normal epithelium and connective tissue fell approximately 50% at 5 and 7 days. In contrast the incorporation of P^{32} in infected tissues was 1.5 to 1.7 times that of normal controls.

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Measurement of Cortical Binding Capacity in Human Plasma.*† (26198)

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The high levels of cortisol in the plasma of pregnant and estrogen-treated patients have been attributed to an α-globulin named transcortin by Slaunwhite and Sandberg(1) and referred to as corticosteroid binding globulin by Daughaday *et al.*(2). These authors have disagreed on whether binding capacity is elevated during pregnancy(1,2,3). These findings have made pertinent the development of procedures to determine reliably and accurately the binding capacity of plasma.

The protein responsible for binding has not been isolated. The binding process has been studied by ultrafiltration(4), single bag equilibrium dialysis at 4°C(1), "two bag" equilibrium dialysis at 4°C(2), and electrophoresis(5,6). Furthermore, Slaunwhite and Sandberg have indicated that the binding is not readily reversible(1). This has broad implication on the physiologic significance of binding in pregnancy and other conditions.

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