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SANDY MCGREGOR for the M.S. in Microbiology
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A quantitative comparison was made of the incorporation of 14, C^{14} lipid compounds into Lee influenza virus. The compounds tested were either lipids reported to be present in the virus or precursors to virus lipid components. Infected chorioallantoic tissues were incubated in medium containing the C^{14} metabolites. The virus was purified by specific adsorption to chicken red blood cells, ultracentrifugation and chromatography on an ECTEOLA anion exchange column. The purified virus was titrated and the C^{14} measured by scintillation counting.

The C^{14} in the tissues was also determined. The incorporation of the compounds into the infected tissues was measured and compared to the uptake by normal tissues.

All but two of the compounds tested were incorporated into the virus in significant amounts. Ethanolamine and choline,

precursors to phospholipids found in the virus, were not incorporated into the virus. This suggested the major virus phospholipids were synthesized before infection. However, glycerol, another phospholipid precursor, was utilized in virus production. It is possible that some of the minor phosphatides of the virus, such as phosphatidic acid, were synthesized after infection.

It appeared that there was a significant decrease in fatty acid synthesis after infection. Only a small amount of malonic acid was incorporated into the virus. Due to their solubility, the fatty acids supplied to the infected host cells were divided into two groups. The long chain acids did not seem to be utilized to a great extent for virus formation. The short chain acids contributed significantly greater amounts of label to the virus, in terms of molar quantities, than any of the long chain acids. The value for butyric acid was greater than for any other fatty acid. In addition, this fatty acid stimulated virus production nearly two fold.

The results suggested that most of the virus cholesterol was preformed host material. There was little incorporation into the virus and infected tissue from the precursor 3-hydroxy-3-methylglutaric acid-3-C^{14}.

The infected tissue incorporated significant amounts of C^{14} from all of the compounds studied. There seemed to be a relation between the amount of C^{14} incorporated into the tissue and that found

in the virus, except in the case of ethanolamine and choline. Both of these compounds were found in the infected tissues in substantial amounts but were not utilized in new virus formation. Results indicated that eight of the fourteen compounds tested were incorporated significantly less in infected tissue than in control tissue.

A QUANTITATIVE COMPARISON OF THE INCORPORATION OF C^{14} LABELED LIPIDS AND LIPID PRECURSORS INTO LEE INFLUENZA VIRUS

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SANDY MCGREGOR

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[redacted]

Professor of Microbiology

In Charge of Major

[redacted]

Chairman of Department of Microbiology

[redacted]

Dean of Graduate School

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TABLE OF CONTENTS

LIST OF TABLES

A QUANTITATIVE COMPARISON OF THE INCORPORATION OF C^{14} LABELED LIPIDS AND LIPID PRECURSORS INTO LEE INFLUENZA VIRUS

INTRODUCTION

The lipid portion of influenza virus has been of particular interest since Hoyle found that the lipid extracted from the purified virus was serologically active and resembled the host cell lipid material (10, p. 242). Since then a question has arisen, that is, was the virus lipid material composed of preformed host cell material or was it newly synthesized material similar to that of the host cell? Frommhagen, Knight and Freeman have also reported that the virus lipid was probably derived from the host cell material (7, p. 195). More recently, Kates et al., using P^{32} labeled calf kidney cells, concluded that the virus lipids are composed of both host material and newly synthesized material (14, p. 456, 464).

There have been several reports of the use of P^{32} to label influenza virus. Hoyle, Jolles and Mitchell were able to account for 20 to 25 percent of the total virus radioactivity in the phospholipids (12). P^{32} labeled virus has been purified and used to follow the virus materials in the infected host cell (11).

In addition to studying some of the lipids reported to be present in the Lee virus it was of interest to determine the contribution to the virus made by certain possible precursors to these compounds.

In order to do this it was necessary to use C^{14} labeled metabolites.

This thesis was a phase of a larger project in which certain C^{14} labeled carbohydrates, amino acids, nucleic acid components and lipids were compared quantitatively for incorporation of C^{14} into Lee influenza virus. All previous results have been reported by Buckley (3).

HISTORICAL REVIEW

Purified Lee strain, type B influenza virus is reported to contain 34. 5 percent lipid material, on a dry weight basis. In addition the virus contains 14 percent ribonucleoprotein (slightly less than one percent RNA), 13 percent mucoprotein (hemagglutinin), 3. 5 percent carbohydrate and 34 percent protein material bound to carbohydrates and lipids (5, p. 210-211).

Frommhagen, Knight and Freeman have done further work on the lipids of the Lee strain and have found the virus to be composed of phospholipid (10. 8 percent), unesterified cholesterol (5. 0 percent) and little or no neutral fats, in terms of dry weight. The phospholipid was made up of cephalin, sphingomyelin, lecithin and a compound with the properties of a glycolipid. In addition they have found there was only 18. 5 percent total lipid extracted by a chloroform-methanol mixture and considered that the remainder reported by Frisch-Niggemeyer and Hoyle could be attributed to protein material. This protein amounted to about 10 percent of the dry virus (6; 7, p. 185, 188-190, 193-194).

In more recent reports the extracted lipid material from the Melbourne strain of type A influenza virus was hydrolyzed and the fatty acid composition determined. The fatty acids found in appreciable quantities were palmitic, linoleic, oleic, stearic, arachidonic

and a C_{22} polyene. Chick embryo-grown virus was found to contain only trace amounts of neutral lipids, but virus grown in calf kidney cells contained mono-, di- and triglycerides, and cholesterol esters. It was also observed that in the case of virus grown in calf kidney cells three compounds were newly synthesized. These were three minor phosphatide components; phosphatidic acid and two other acid phosphatides which were not identified. The amount of these phosphatides synthesized during infection varied; however the total amount of phosphatides in the virus remained constant. It was noted that there was a close similarity of the virus lipid composition to that of the host cell, especially in the case of the fatty acids. This information was interpreted to mean the virus lipid was composed of both host cell material and newly-synthesized materials (14, p. 458-459, 462-465).

MATERIALS AND METHODS

A number of lipid compounds reported to be present in Lee influenza virus or thought to be precursors of these compounds were selected and tested for toxicity. This preliminary work consisted of testing the compounds at three concentrations, 8 mM., 4 mM., and 2 mM. in the tissue culture medium. The compounds were not only tested for their effect on the virus yield but also for the solubility at these concentrations. Some were not soluble at these levels and therefore had to be run at lower concentrations. After consideration of toxicity, solubility, the amount reported to be present in the virus and the availability of the C^{14} labeled compound, 12 compounds were selected to be tested for incorporation into the virus.

For each C^{14} labeled compound, two duplicate experiments were run to determine the amount of the compound, or a part thereof, incorporated into the virus particle. Chorioallantoic tissues infected with the virus were incubated 44 hours in the medium containing the compound in a concentration of either 8 mM. or 1 mM. In each case the total radioactivity was held at about 10^6 CPM per milliliter.[1] The virus was purified, titrated and the radioactivity counted as described below. In addition, the infected tissues were used to

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[1] Absolute CPM, corrected for counting efficiency.

measure the uptake of the compound by the host cell. The tissues were dried in screw cap vials and dissolved in a Hyamine solution for determination of CPM per milligram. The method used was described by Buckley (3, p. 39, 49).

In addition to the virus experiments, one experiment was run with each compound to compare the amount of incorporation into infected and non-infected tissues. For each compound there were two groups of cultures, one infected and the other non-infected. The total radioactivity in the medium was 2×10^5 CPM per milliliter. After incubation the tissues from all infected cultures were randomly mixed and divided into two samples for C^{14} measurement. Tissues from non-infected cultures were pooled and treated in a similar manner.

Medium

The medium used in all of the experiments was buffered glucosol (9). The complete medium consisted of a mixture of equal volumes of two separate solutions, a phosphate buffer and a glucose-salts solution. These were prepared as follows:

Buffer

Na_2HPO_4 (anhydrous)	7.105 gm.
KH_2PO_4 (anhydrous)	2.269 gm.

Phenol Red	0. 020 gm.

Dilute to one liter with deionized water.

Glucose-salts

NaCl	8. 000 gm.
$CaCl_2 . 2H_2O$	0. 265 gm.
$MgCl_2 . 6H_2O$	0. 500 gm.
Glucose (anhydrous)	1. 440 gm.

Dilute to one liter with deionized water.

Each solution was dispensed in 150 milliliter screw cap bottles (100 ml. per bottle), stoppered with cotton and gauze and closed with rubber lined caps. The solutions were sterilized by autoclaving at 121° C. for 15 minutes. The pH of the sterile buffered glucosol was between 7. 2 and 7. 3.

Penicillin G and streptomycin sulfate were added to the combined medium just before use to obtain a final concentration of ten units per milliliter and 40 micrograms per milliliter respectively.

Virus

The virus used in all experimental work was the Lee strain of type B influenza virus, termed Lee-R, and was supplied to this department by Dr. F. L. Horsfall of the Rockefeller Institute. This strain has been passed numerous times in embryonated eggs.

Stock virus, consisting of allantoic fluid from infected eggs,

was prepared and stored at -60° C. as described by Bowen (2, p. 5-6).

Eggs

Fertile, New Hampshire Red eggs were obtained from the Mid-Valley Hatchery and used as a source of chorioallantoic membranes and in the preparation of stock virus. Eggs were incubated 10 to 11 days at 38° C. in a humid atmosphere before being used.

Chemicals

All chemicals used in the preparation of the medium and other solutions were reagent grade. The compounds used in the preliminary testing were of the purest grade available.

The C^{14} labeled compounds were obtained from the following sources: glycerol-UL-C^{14}, choline chloride-methyl-C^{14}, ethanolamine-1,2-C^{14}, HCL, oleic acid-1-C^{14}, sodium acetate-1,2-C^{14} and malonic acid-1,3-C^{14} from Nichem Incorp.; benzoic acid-C^{14} (standard) and 3-OH-3-CH_3-glutaric acid-3-C^{14} from New England Nuclear; palmitic acid-1-C^{14} and stearic acid-1-C^{14} from Tracerlab; sodium valerate-1-C^{14}, sodium caproate-1-C^{14} and sodium butyrate-1-C^{14} from California Biochemical Co.; linoleic acid-1-C^{14} and cholesterol-4-C^{14} from Nuclear-Chicago Corp. Table 1 shows a comparison of the specific activities as listed by the manufacturer

Table 1. A comparison of specific activities of C^{14} labeled compounds.

Compound	Specific Activity (μcuries per μmole) Listed on manufacturer's label	Determined by scintillation counting[a]
Glycerol-UL-C^{14}	26.32	16.90
Choline chloride-methyl-C^{14}	7.16	4.23
Ethanolamine-1,2-C^{14}	0.33	0.33
Oleic acid-1-C^{14}	10.00	9.45
Sodium acetate-1,2-C^{14}	1.00	0.50
Malonic acid-1,3-C^{14}	0.50	0.35
3-hydroxy-3-methyl glutaric acid-3-C^{14}	2.37	1.43
Palmitic acid-1-C^{14}	6.23	6.20
Stearic acid-1-C^{14}	1.12	0.92
Sodium valerate-1-C^{14}	0.88	0.75
Sodium caproate-1-C^{14}	5.00	5.15
Sodium butyrate-1-C^{14}	1.54	1.24
Linoleic acid-1-C^{14}	24.70	36.40
Cholesterol-4-C^{14}	19.40	20.20

[a]This value was determined as follows: The absolute CPM/ml. of a stock solution was determined for five samples as described below. From this a mean value was obtained. The milligrams of compound per milliliter of stock solution was known and the micromoles per milliliter was calculated.

$$\frac{\text{Absolute CPM/ml.}}{\mu\text{moles/ml.}} = \text{CPM}/\mu\text{mole}$$

The microcurie is defined as 2.22×10^6 disintegrations per minute (13, p. 404).

$$\frac{\text{CPM}/\mu\text{mole}}{2.22 \times 10^6 \text{ CPM}/\mu\text{curie}} = \text{Specific activity } (\mu\text{curies}/\mu\text{mole})$$

and as determined experimentally in this laboratory. It is evident that there are some large discrepancies. Our own data, obtained by scintillation counting, were used in all calculations.

Tissue Culture Techniques

The cultures were set up by Buckley's modification of the method of Tamm, Folkers and Horsfall (3, p. 35-39).

Most of the compounds tested were run at a concentration of eight millimolar. Palmitic acid, stearic acid, oleic acid, linoleic acid and cholesterol, due to their low water solubility, were run at a concentration of one millimolar.

Stock solutions of the compounds used at eight millimolar were prepared as follows: The dry weight of the compound was first determined by obtaining the gross weight of the opened vial and compound after drying in vacuuo over P_2O_5 overnight. The compound was then dissolved in a small volume of the buffer portion of the medium and transferred to a volumetric flask together with five rinses from the vial. The latter was then dried and weighed. The pH of the solution was adjusted to approximately 7.2 with either 1 N HCl or 1 N NaOH solutions. The solution was diluted with buffer to a concentration of two microcuries per milliliter using the manufacturer's value for total activity. After sterilization by sintered glass filtration the stock was stored at 4 to 6° C. in a screw cap

bottle.

For the five relatively insoluble compounds a different system had to be devised. Attempts to use oils and an emulsifying agent (Tween 80), individually and in combination, failed due to toxicity. Shapiro, Chowers and Rose have reported that the presence of five percent bovine or human albumin favored the solubility of certain long chain fatty acids (16). It was found that solutions or stable emulsions of the five compounds could be prepared at a concentration of one millimolar by incorporating five percent bovine albumin into the medium. The following procedure was used: The dry weight of the compound was determined as above except that the compound was dissolved in either petroleum ether or chloroform instead of buffer, at a concentration of two microcuries per milliliter. The solution was then distributed in ten ml. volumetric flasks with glass stoppers and stored at -60° C. A Virtis homogenizer was used to disperse the compound into the aqueous medium. The buffer portion of the medium (usually 15 ml.) was added to a sterile, 100 ml. Virtis container fitted with a teflon lid and stainless-steel blade assembly. The solvent, containing the C^{14} compound, was added to the Virtis container along with enough solvent containing the unlabeled compound to bring the concentration to the desired level. The total volume of solvent varied due to the different specific activities of the stock solutions, but was usually less than ten ml.

The container, with the lid ajar, was heated at 60° C. for about ten minutes until most of the solvent was gone or until the compound started to precipitate. The mixture was homogenized immediately at a moderate speed (Virtis rheostat setting of 10-15) for two minutes. The heating was continued until all of the solvent was removed. Experiments showed that after 15 minutes there was no solvent remaining. The homogenization was then repeated for one minute. The emulsion was cooled rapidly and the glucose-salts portion of the medium (15 ml.), containing ten percent bovine albumin (pH 7.0), added. The medium was homogenized at the slowest possible speed for 30 to 60 seconds. After addition of the antibiotics in the concentrations previously mentioned, the medium was placed on a rotary shaker (160 cycles per minute) for at least one hour at 35° C. This procedure yielded a clear solution for all of the five compounds but cholesterol which formed a stable emulsion.

To determine the exact specific activity of the stock solutions, either the buffer or solvent solution, 0.5 ml. of the stock solution was diluted with deionized water and sufficient medium to give a final dilution of the stock of 1:100 and of the medium of 1:25. In order to dilute the stock solutions prepared with organic solvents, the procedure outlined above for dispersing the water insoluble compounds in the aqueous medium was used. Five, 0.5 ml. samples of the diluted stock were accurately pipetted into counting vials and

the CPM per milliliter were determined as described below. Using this information the culture medium could be prepared at a known specific activity. The concentration of the compound was adjusted with a solution of the unlabeled compound.

Purification of the Virus

Purification of the virus was accomplished by a three-step process. This involved (1) adsorption onto and elution from chicken red blood cells, (2) ultracentrifugation and resuspension and (3) chromatography on an ECTEOLA cellulose anion exchange column. The procedure was a modification of Buckley's method (3, p. 39-44).

The clarification of the tissue culture fluid and the first step of the purification have not been changed.

The ultracentrifugation procedure has been slightly modified. In order to get a more complete sedimentation of the virus, the speed was increased to 30, 000 RPM (approximately 59, 300 x g) for 45 minutes. This treatment eliminated most of the stir-back effect. The supernatant was removed and the virus resuspended in 0. 02 M phosphate buffer at pH 7. 0 as described by Buckley.

The ECTEOLA columns were prepared as described except pressure, applied by an electric pump, was used in place of vacuum to facilitate flow. Washing of the column with 1 N HCl, 1 N NaOH, 0. 5 M NaCl and 0. 02 M phosphate buffer (pH 6. 4) was effected with

about 47 mm. of Hg pressure. The "precolumn" virus material was placed on the washed column (25 mm. of Hg pressure), leaving only enough for titration and radioactivity measurement. A total of ten fractions were collected from the column. Fraction 0 was the volume collected as the virus sample was placed on the column. Fractions 1 through 4 were washes of 0. 02 M phosphate buffer at pH 7. 0 and had a total volume of 38 ml. After addition of 0. 02 M phosphate buffer containing 0. 5 M NaCl, fractions 5 through 9 were collected. They had volumes of 1. 5 ml., 2. 6 ml., 3. 0 ml., 3. 0 ml. and 5. 0 ml. respectively. The virus was found in fractions 6, 7, 8 and 9. Fraction 6 contained most of the virus and was the purest preparation.

Titration of the Virus

The virus titer was determined by a method similar to Salk's hemagglutination method (3, p. 24-26). Two series of two fold virus dilutions were prepared in physiological saline. One started with a 1:4 dilution and the second with a 1:6 dilution. Each series was run in duplicate in most cases. An equal volume of a 0. 5 percent suspension of washed chicken red blood cells was added to each tube and the mixture incubated for 35 minutes at room temperature. The titer was read as the reciprocal of the dilution of the last tube showing complete hemagglutination. The titer was expressed as

hemagglutinating units per milliliter (HA units per ml.).

Radioactivity Measurements

All counting was done with an automatic, model 314, Tri-Carb liquid scintillation spectrometer. The samples were suspended in a Cab-O-Sil gel system (1, p. 96-100), and counted in Wheaton counting vials. This procedure has been described by Buckley (3, p. 45-49).

Samples from all of the ECTEOLA column fractions were counted. A 2.0 ml. sample from each fraction, except fraction 5, was accurately pipetted into counting vials. Since fraction 5 had such a small volume, the sample was reduced to 1.0 ml. These were dried in vacuuo, over P_2O_5 at 40° C. for 48 hours. The sample was then dissolved in 0.5 ml. of a 1:25 dilution of the medium and 14.5 ml. of Cab-O-Sil gel was added. All other virus fractions, the Spinco supernatant and the "precolumn" material, were counted in the same manner using 0.5 ml. samples.

One, 0.5 ml. sample of the diluted tissue material from each screw cap vial was counted.

For most of the measurements four counts on each sample were used to determine the mean counts per minute. It was later found that three counts on each vial were sufficient. On all counts the vials were incubated at 0° C. in the dark for 24 hours prior to

counting, and after an internal standard was added the vials were incubated under the same conditions for three days before counting.

Since the different samples and compounds were counted at different efficiencies a correction to 100 percent efficiency was necessary. Each sample was first counted to determine the mean CPM. Then an internal standard of benzoic acid-C^{14} was added. The sample was recounted and the increase in CPM caused by the internal standard determined. This increase in CPM was divided by the theoretical CPM of the added benzoic acid to determine the efficiency. The mean CPM of the sample was then divided by the efficiency to give the corrected CPM. All counts in this thesis represent corrected values.

Statistical Methods

The least significant difference of the means of paired, virus incorporation experiments was determined by Student's "t" test. This same method was used to determine the least significant difference between the mean values for incorporation of different compounds into infected and non-infected tissues (15, p. 137-140, 146-147, 206-207, 211; 4, p. 121-122, 174).

EXPERIMENTAL RESULTS

In the selection of the compounds to be studied, it was decided that the precursors to the phospholipids would be of greatest interest since they are found in considerable amount in the virus. There are several different types of compounds which could be precursors to the phospholipids.

Ethanolamine and choline in a phosphorylated form, can combine with a diglyceride to form phosphatidyl ethanolamine and lecithin respectively (8, p. 566-568). Both phosphatidyl ethanolamine and lecithin are reported to be found in the virus in considerable amounts (7, p. 189-190).

In addition to their use in the synthesis of cephalins and lecithins, glycerides are utilized in the formation of phosphatidic acids, a minor phosphatide found in the virus. Therefore glycerol was selected for testing.

The only other major component of phospholipids is the fatty acid. Oleic, palmitic, stearic, linoleic and arachidonic acids are reported to be found in ester linkages with the phospholipids (8, p. 566-567). All of these acids were also found in the lipid extracted from the virus (14, p. 459). All of them except arachidonic acid were studied.

Since the long chain fatty acids were tested it was also of

interest to examine some of the short chain acids. These could contribute either to the lipid portion or could be modified and used for synthesis of other virus fractions. After preliminary testing a C_4, C_5 and C_6 acid were selected for study.

Cholesterol, found in the virus in large amounts, was studied along with a single precursor. Several precursors were tested on a preliminary basis but 3-hydroxy-3-methylglutaric acid was the only precursor available in the labeled form.

The compounds cannot be compared as a whole, but must be separated into two groups; those run at eight millimolar concentration and those run at one millimolar. However, some relations can be observed between those compounds run at different concentrations. The values for the incorporation of C^{14} from the compounds, shown in Table 2, are expressed in terms of the number of micromoles of each compound that have contributed their C^{14} to 10^6 HA units of virus. These data were statistically analyzed in Table 3. It was found that the means from duplicate experiments must differ more than $\pm$ 1.59 micromoles per 10^6 hemagglutinating units to be significant.

Fatty Acids and Precursors

Since fatty acids are reportedly found in considerable amount in the virus phospholipid, presumably in an ester form, and are also

Table 2. Quantitative comparison of C^{14} incorporation from labeled lipid metabolites and precursors into Lee influenza virus, developing in tissue culture.

Labeled Compound	Micromoles of compound/10^6 HA units of virus[c, e]
Palmitic acid-1-C^{14} [a]	0.87
Stearic acid-1-C^{14} [a]	0.77
Linoleic acid-1-C^{14} [a]	0.57
Oleic acid-1-C^{14} [a]	0.64
Cholesterol-4-C^{14} [a]	3.25
3-hydroxy-3-methylglutaric acid-3-C^{14}	0.59
Sodium butyrate-1-C^{14}	5.88
Sodium valerate-1-C^{14}	4.43
Sodium caproate-1-C^{14}	3.40
Malonic acid-1, 3-C^{14}	1.14
Sodium acetate-1, 2-C^{14}	21.2
Ethanolamine-1, 2-C^{14}	1.25 [d]
Choline chloride-methyl-C^{14} [b]	0.27 [d]
Glycerol-UL-C^{14} [b]	2.20

[a] These compounds were tested at 1 millimolar concentration. All others were tested at 8 millimolar.

[b] Data for these two compounds were taken from Buckley's work.

[c] Least significant differences between two values in table was found to be $\pm 1.59\,\mu$moles/10^6 HA units.

[d] Not considered significant since the CPM per milliliter of sample was less than 5X background.

[e] Values are means of duplicate experiments. Each individual value was calculated as outlined in the following example. The results used are for butyric acid. The uncorrected CPM for the 2.0 ml. virus sample were 1091, 1038 and 1065. The sample contained 1120 HA units per milliliter.

Average uncorrected CPM = 1065

The CPM due to the background was found to be 22. This meant only 1043 CPM were from the virus sample. The efficiency of counting was determined, as described above, and found to be 41.3 percent for the 2.0 ml. sample.

Corrected CPM/milliliter = 1263

$$\frac{1263 \text{ CPM/milliliter}}{1120 \text{ HA units/milliliter}} = 1.128 \text{ CPM/HA unit}$$

$$= 1.128 \times 10^6 \text{ CPM}/10^6 \text{ HA units}$$

$$\frac{1.128 \times 10^6 \text{ CPM}/10^6 \text{ HA units}}{0.1688 \times 10^6 \text{ CPM}/\mu\text{mole of butyric}} = 6.69\,\mu \text{ moles of butyric}/10^6 \text{ HA units}$$

Table 3. Statistical analysis of values from duplicate experiments measuring the C^{14} incorporation into Lee influenza virus.

Compound	Expt. A	Expt. B	Difference	$(\text{Difference})^2$
Palmitic	0.82	0.92	0.10	0.01
Stearic	0.99	0.56	0.43	0.18
Sodium acetate	20.0	22.40	2.40	5.76
Ethanolamine	1.50	1.00	0.50	0.25
Sodium caproate	4.35	2.45	1.90	3.61
Sodium valerate	5.08	3.78	1.30	1.69
Sodium butyrate	6.69	5.06	1.63	2.66
Cholesterol	2.81	3.69	0.88	0.77
Linoleic	0.57	0.56	0.01	0.00
Malonic	1.13	1.15	0.02	0.00
Oleic	0.73	0.55	0.18	0.03
Hydroxymethylglutaric	0.75	0.44	0.31	0.10
Glycerol	2.00	2.40	0.40	0.16
Choline	0.33	0.21	0.12	0.01
			Sum	15.23

Number of pairs = 14 Divisor = 28

$$\text{Variance} = \frac{\text{Sum of the (differences)}^2}{28} = \frac{15.23}{28} = 0.5439$$

$$\text{Standard deviation} = \pm\sqrt{\text{variance}} = \pm\sqrt{0.5439} = \pm 0.7375$$

$$\text{Standard error of the difference between single measurements} = \pm\sqrt{(\text{S.D.})^2 + (\text{S.D.})^2}$$

$$= \pm\sqrt{2 \times 0.5439}$$

$$= \pm\sqrt{1.0878}$$

$$\doteq \pm\ 1.0430$$

"t" (P = 0.05 and n = 13) = 2.16

"t" x S.E. = 2.16 x $\pm$ 1.0430 = $\pm$ 2.2529 μ moles/10^6 HA units.

Standard error of the difference between the means of duplicate experiments.

$$= \pm\sqrt{\frac{(\text{S.D.})^2}{2} + \frac{(\text{S.D.})^2}{2}}$$

$$= \pm\sqrt{\frac{2 \times 0.5439}{2}}$$

$$= \pm\ 0.7375$$

"t" x S.E. = 2.16 x $\pm$ 0.7375 = $\pm$ 1.59 μ moles/10^6 HA units.

Thus the difference between two values in Table 2, each of which is the mean of duplicate experiments, must be greater than $\pm$ 1.59 to be significant.

thought to be precursors of more complex compounds such as sphingomyelin, it was felt that this was an important group to be tested.

The results showed that the long chain fatty acids contributed only a small amount of label to the virus, in terms of micromoles of compound, as compared with some of the other compounds in the table. However, if these molecules were incorporated intact, their total contribution in terms of carbon atoms is considerable. The amounts of the two saturated acids incorporated did not differ significantly from those of the unsaturated acids. Even though the long chain acids were tested at only one millimolar, it must be noted that this concentration represented about the maximum that could be held in solution by the albumin.

Although there can be no direct comparison with the long chain acids, the short chain fatty acids made a significantly greater contribution to the virus on a molar basis than any of the long chain acids. There are some differences in the values from the C_4, C_5 and C_6 acids, but the only significant difference was between butyric and caproic acids. The results suggested that the amount of incorporation was inversely related to the chain length.

Butyric acid proved to be one of the most interesting compounds tested. In preliminary experiments it was noted that this compound at concentrations ranging from 1 to 16 millimolar

stimulated virus production. Statistical analysis of nine experiments with butyric acid at a concentration of eight millimolar showed that there was a significant difference between cultures containing the fatty acid and control cultures. Butyric acid caused a 1.7 fold stimulation of virus production over the control. These data are presented in Table 4.

Malonic acid, a reported precursor to fatty acid synthesis contributed a small amount of label to the virus. The value (Table 2) was significantly less than any of the short chain acids.

Cholesterol and Precursor

It would be of interest to determine whether or not the cholesterol of the virus was preformed, synthesized during the infection or a combination of both. In the hope of shedding some light on this question cholesterol-4-C^{14} and a reported precursor, 3-hydroxy-3-methylglutaric acid-3-C^{14}, were studied. Since the cholesterol was tested at one millimolar and the glutaric acid derivative at eight millimolar it was difficult to compare them. However, in view of the results obtained, this concentration difference did not appear important.

The mean value for the micromoles of cholesterol incorporated (Table 2) was much greater than any of the long chain fatty acids which were at comparable concentrations. The value was not significantly

Table 4. A statistical comparison of the virus production in medium containing 8 millimolar butyric acid and in control medium.

Experiment number	HA titer in culture fluid	Log_{10} of the HA titer	Deviation from mean	$(Deviation)^2$
Butyric acid				
1	2048	3.31133	0.50892	0.25900
2	512	2.70927	0.09314	0.00868
3	512	2.70927	0.09314	0.00868
4	768	2.88536	0.08295	0.00688
5	896	2.95231	0.14990	0.02247
6	512	2.70927	0.09314	0.00868
7	448	2.65128	0.15113	0.02284
8	384	2.58433	0.21808	0.04756
9	512	2.70927	0.09314	0.00868
		25.22169		0.38347
Control				
1	512	2.70927	0.09892	0.00979
2	320	2.50515	0.10520	0.01107
3	320	2.50515	0.10520	0.01107
4	768	2.88536	0.27501	0.07563
5	512	2.70927	0.09892	0.00979
6	320	2.50515	0.10520	0.01107
7	384	2.58433	0.02602	0.00068
8	384	2.58433	0.02602	0.00068
9	320	2.50515	0.10520	0.01107
		23.49316		0.14085

Mean of Butyric acid = 2.80241
Mean of Control = 2.61035

Combined sum of the $(deviation)^2$ = 0.52432
Sum of the degrees of freedom = (9-1) + (9-1) =16

$$\text{Standard deviation} = \pm\sqrt{\frac{\text{Combined sum of the (deviation)}^2}{\text{Sum of the degrees of freedom}}} = \pm\sqrt{\frac{0.52432}{16}} = \pm 0.18103$$

$$\text{Standard error of the difference between the means} = \pm\sqrt{\frac{(0.18103)^2}{9} + \frac{(0.18103)^2}{9}}$$

$$= \pm\sqrt{0.00728}$$

$$= \pm 0.08530$$

Difference of the means = 0.19206

$$\frac{\text{Difference of the means}}{\text{Standard error of the difference}} = \text{"t"} = \frac{0.19206}{0.08530} = 2.25$$

Fisher's value for "t" (P = 0.05 and n = 16) = 2.12

Since the value for "t" determined above was greater than expected from chance variation in the data, the medium containing butyric acid produced a significant stimulation in virus production over that of the control medium (4. p. 121-122, 174).

different from two of the short chain fatty acids, but was lower than butyric acid. However, one must consider the total carbon in each compound. If cholesterol was incorporated unchanged, this would represent over three times more carbon than any of the short chain acids. In any case these results suggest that a considerable amount of cholesterol from the medium was utilized in virus synthesis.

There was very little C^{14} incorporation into the virus from the glutaric acid derivative. This suggests that little if any cholesterol synthesis took place during the experiment, and that the virus cholesterol was probably derived from the preformed supply in the cell.

It would have been desirable to study other cholesterol precursors, but none were available in the C^{14} labeled form.

Possible Precursors to Phospholipids

Among the three compounds tested (ethanolamine, choline and glycerol) in this group, two were encountered which incorporated so little C^{14} that counts per minute in the isolated virus sample were less than five times background. These values were considered insignificant. It was interesting to note that even though little or no C^{14} was incorporated into the virus, considerable amounts were taken up by the tissues (Table 5). Ethanolamine and choline were the only two compounds found of which this was true. Their failure

Table 5. A statistical analysis of duplicate experiments measuring the C^{14} incorporation into chorioallantoic tissue infected with Lee influenza virus.

Compound	Micromoles/100 milligrams dry tissue A	B	Difference	(Difference)^2	Mean of A & B
Palmitic	6.5	6.1	0.4	0.16	6.3
Stearic	4.5	3.9	0.6	0.36	4.2
Acetate	34.3	47.5	13.2	174.24	40.9
Ethanolamine	20.3	23.4	3.1	9.61	21.9
Caproic	9.0	5.1	3.9	15.21	7.2
Valeric	10.8	7.9	2.9	8.41	9.4
Butyric	9.8	7.7	2.1	4.41	8.8
Cholesterol	11.4	31.1	19.7	388.09	21.3
Linoleic	5.1	5.1	0.0	0.00	5.1
Malonic	3.4	3.8	0.4	0.16	3.6
Oleic	7.0	7.1	0.1	0.01	7.1
Hydroxymethyl glutaric	1.2	1.6	0.4	0.16	1.4
Choline	16.8	15.9	0.9	0.81	16.4
Glycerol	13.1	13.5	0.4	0.16	13.3
				601.79	

Number of pairs = 14 Divisor = 28

$$\text{Variance} = \frac{\text{Sum of the (difference)}^2}{28} = \frac{601.79}{28} = 21.4925$$

$$\text{Standard deviation} = \pm\sqrt{21.4925} = \pm 4.6360$$

$$\text{Standard error of the difference of a single measurement} = \pm\sqrt{(\text{S. D.})^2 + (\text{S D.})^2} = \pm\sqrt{2 \times 21.4925}$$
$$= \pm 6.5563$$

"t" (P = 0.05 and n = 13) = 2.16

"t" x S E. = 2.16 x $\pm$ 6.5563 = $\pm$ 14.162 μmoles/100 milligrams dry tissue.

$$\text{S E. of the difference of the means} = \pm\sqrt{\frac{(\text{S. D})^2}{2} + \frac{(\text{S. D.})^2}{2}}$$
$$= \pm\sqrt{\frac{2 \times 21.4925}{2}} = \pm 4.6360$$

"t" x S.E = 2.16 x $\pm$ 4.6360 = $\pm$ 10.01 μmoles/100 milligrams dry tissue.

to be incorporated into the virus in these experiments demonstrates the selective character of metabolite assimilation in virus synthesis. Labeled metabolites are utilized in different amounts; some are not used at all or only in trace amounts.

Even though two of the precursors to phospholipids gave insignificant incorporation, glycerol had a value comparable to some of the short chain fatty acids. Its actual disposition within the virus is of course unknown.

Acetate

Acetate was tested with the lipids because it could be incorporated into all of the reported lipid fractions in the virus. However, it could also be incorporated into nearly all of the non-lipid fractions. Therefore the value for acetate was not too meaningful with respect to any single fraction. It was of interest because the micromoles incorporated into the virus was greater than for any other compound tested. This included carbohydrates, amino acids, nucleic acids and lipids. This finding would be expected if acetate in the intracellular pool were being utilized in the synthesis of a variety of metabolites which were then being utilized for new virus formation.

Incorporation of C^{14} Compounds into Infected Host Tissue

It was also necessary to determine quantitatively the uptake of

the labeled metabolites by the host tissue, as an indication of their availability for viral synthesis, and to provide a quantitative comparison of incorporation into tissue and virus. To accomplish this, not only was the incorporation into the virus determined, but also the incorporation into the infected chorioallantoic tissue. Data for infected tissues from the virus experiments, together with a statistical analysis of them are given in Table 5.

The least significant difference between mean values of duplicate experiments was $\pm$10. 0 micromoles per 100 milligrams of dry tissue.

These data are of greatest interest when compared with the parallel values for the virus. It is evident that in both host cells and virus, acetate had by far the highest molar incorporation value, indicating it was probably used freely by the cell in biosynthesis of metabolites which then appeared in considerable amounts in the virus. The sodium acetate taken up by the host cells amounted to about 3. 36 percent of the cell material (dry weight basis).

The compound showing the second highest molar uptake by the cells was ethanolamine. Despite this fact, it was used to only a very slight extent in the virus. Choline was also absorbed abundantly by the cells but did not contribute significant amounts of C^{14} to the virus.

Cholesterol was taken up in relatively large molar quantities

by the host cells and this was reflected in a comparable contribution to the virus.

Glycerol and the C_4, C_5 and C_6 fatty acids formed a group showing moderately large molar uptake by the tissues, with no significant differences between values. However, in the case of the virus, the contribution of these compounds showed differences. Glycerol made the smallest contribution to the virus and sodium butyrate the largest. The value for both butyrate and valerate were significantly higher than glycerol. The high values for butyrate may be related to its stimulatory effect on virus synthesis. It may supply a factor which is present in a limiting concentration in the cell.

The long chain fatty acids were also taken up by the host cell in moderately large molar quantity, not significantly different from the shorter chain acids. However, the molar contribution of C^{14} from these compounds to the virus was in all cases significantly lower than for the C_4, C_5 and C_6 acids. These results suggest a more limited disposition of the long chain acids in the virus. It is possible that they are confined to the lipid fraction while the shorter chain acids might follow more than one metabolic pathway into the virus.

The compound showing the lowest uptake by the tissue was 3-hydroxy-3-methylglutaric acid. No particular reason for this was

evident, but it would be expected to result in a very small contribution of this compound to the virus, as was found to be the case.

Comparison of the Incorporation of C^{14} Compounds into Infected and Control Tissues

In addition to determining the amount of incorporation into infected tissues, it was of interest to compare this amount to that incorporated into non-infected tissues. Table 6 lists the values for an infected and a control group for each compound. Each value represents the mean of two samples taken from a single experiment.

A statistical analysis for the least significant difference between the means of the infected and control groups is given in Table 7. The difference was considered significant if the values from infected and normal cultures in the same experiment, testing a single compound, differed more than ± 2.67 micromoles per 100 milligrams of dry tissue. This is true only if each value is the mean from duplicate tissue samples. The least significant difference found in this table is much lower than that in Table 5 because the data do not include the factor of variation between completely separate experiments. The differences used are only those between duplicate samples within a single experiment. Therefore, the measure of the least significant difference cannot be applied to the results of two different experiments.

Table 6. A comparison of the amount of C^{14} incorporation into Lee influenza virus infected chorioallantoic tissues and control chorioallantoic tissues.

Compound	Micromoles/100 milligram dry tissue[d] Infected	Control	Difference: Control minus Infected[c]
Palmitic acid-1-C^{14} [a]	6.3	7.3	1.0
Stearic acid-1-C^{14} [a]	4.5	5.0	0.5
Sodium acetate-1,2-C^{14}	36.6	45.1	8.5
Ethanolamine-1,2-C^{14}, HCl	31.3	43.6	12.3
Sodium caproate-1-C^{14}	6.9	10.5	3.6
Sodium valerate-1-C^{14}	7.7	14.1	6.4
Sodium butyrate-1-C^{14}	11.1	12.9	1.8
Cholesterol-4-C^{14} [a]	9.8	10.6	0.8
Linoleic acid-1-C^{14} [a]	4.7	6.0	1.3
Malonic acid-1,3-C^{14}	4.2	7.3	3.1
Oleic acid-1-C^{14} [a]	7.3	9.1	1.8
Hydroxymethylglutaric-3-C^{14}	1.5	5.9	4.4
Glycerol-UL-C^{14} [b]	12.7	19.7	7.0
Choline chloride-methyl-C^{14} [b]	21.8	28.0	6.2

[a]Tested at a concentration of 1 millimolar.

[b]Data obtained from Buckley (1).

[c]Least significant difference was $\pm$ 2.67 μmoles/100 milligrams dry tissue (Table 7).

[d]Each value in table represents the mean obtained from two samples of tissue taken from a single experiment. The medium contained about 2×10^5 CPM/milliliter.

Table 7. A statistical analysis of data showing C^{14} incorporation into Lee influenza virus infected chorioallantoic tissues and control chorioallantoic tissues using duplicate samples from a single experiment.

Compound		Micromoles/100 milligrams dry tissue A	B	Difference	(Difference)^2
Palmitic	Infected	6.2	6.5	0.3	0.09
	Control	7.7	6.9	0.8	0.64
Stearic	Infected	4.2	4.8	0.6	0.36
	Control	5.0	5.0	0.0	0.00
Acetate	Infected	37.2	35.8	1.4	1.96
	Control	44.1	46.0	1.9	3.61
Ethanolamine	Infected	31.2	31.4	0.2	0.04
	Control	43.8	43.4	0.4	0.16
Caproic	Infected	6.3	7.5	1.2	1.44
	Control	10.0	11.1	1.1	1.21
Valeric	Infected	7.8	7.5	0.3	0.09
	Control	14.7	13.4	1.3	1.69
Butyric	Infected	9.6	12.7	3.1	9.61
	Control	12.9	12.9	0.0	0.00
Cholesterol	Infected	9.9	9.8	0.1	0.01
	Control	8.0	13.3	5.3	28.09
Linoleic	Infected	4.8	4.6	0.2	0.04
	Control	5.6	6.4	0.8	0.64
Malonic	Infected	4.2	4.1	0.1	0.01
	Control	7.8	6.8	1.0	1.00
Oleic	Infected	7.4	7.1	0.3	0.09
	Control	8.6	9.6	1.0	1.00
Hydroxymethyl-glutaric	Infected	1.8	1.2	0.6	0.36
	Control	6.1	5.8	0.3	0.09
Glycerol	Infected	13.2	12.2	1.0	1.00
	Control	20.2	19.2	1.0	1.00
Choline	Infected	24.7	18.8	5.9	34.81
	Control	28.7	27.2	1.5	2.25
					91.29

Number of pairs = 28 Variance = $\frac{\text{Sum of the (difference)}^2}{56}$

Standard deviation = $\pm\sqrt{1.6302} = \pm 1.2768$

Standard error of the difference of single measurements $= \pm\sqrt{(\text{S. D.})^2 + (\text{S D.})^2}$

$= \pm\sqrt{2 \times 1.6302}$

$= \pm 1.8057$

"t" (P = 0.05 and n = 27) = 2.052

"t" x S.E. = 2.052 x $\pm$ 1.8057 = $\pm$ 3.705 μmoles/100 milligrams dry tissue.

S.E. of the difference of the means = $\pm\sqrt{\frac{(S.D.)^2}{2} + \frac{(S.D.)^2}{2}}$

$= \pm\sqrt{\frac{2 \times 1.6302}{2}}$

$= \pm$ 1.2768

"t" x S.E. = 2.052 x $\pm$ 1.2768 = $\pm$ 2.620 μmoles/100 milligrams dry tissue.

In the case of eight of fourteen compounds, the molar quantities retained in the tissues at the end of the incubation period were significantly lower in infected cultures than in controls. This is in agreement with the findings of Buckley for amino acids and carbohydrates (3, p. 134, 140). It may be due to more rapid leakage through the membranes of the infected cells.

It is interesting to note that most of the compounds showing insignificant differences between infected and control tissues, were those having low water solubility, e. g. the long chain fatty acids and cholesterol. These compounds may be more completely bound to proteins or other large molecules in the cell. Sodium butyrate also showed an insignificant difference between infected and control cells, which could be associated with its apparent high incorporation value in the virus.

DISCUSSION

The result showed that all but two of the compounds tested contributed significant amounts of C^{14} to the virus, and in some cases at least, were probably incorporated directly into the virus. The C^{14} incorporation could involve the whole molecule as a part of a larger molecule, part of the molecule as a part of a larger molecule and in some cases such as cholesterol, probably the whole molecule in an unmodified form.

Ethanolamine and choline, precursors to phospholipids reported to be present in the virus, appeared to be selectively ignored in the synthesis of virus materials. This was not a permeability factor since considerable amounts were found in the infected tissues. There are several possible explanations. Kates, et al. have shown that the major phosphatides in the virus, of which ethanolamine and choline are precursors, were not synthesized after infection. Our data would tend to support this explanation. If the enzymes necessary for the phosphorylation of ethanolamine and choline were inactivated or the cytidine co-enzymes were unavailable after infection then the synthesis of lecithin and phosphatidyl ethanolamine would stop (14, p. 463, 465). There was significant C^{14} incorporation into the virus from labeled glycerol. By conversion to phosphatidic acid, it would be possible for glycerol to be used for virus

synthesis. It has been reported that two unidentified phosphatides and phosphatidic acid were the only phospholipids found in the virus which were synthesized after infection (14, p. 463-465). However, it was also possible for the glycerol to be oxidized to dihydroxyacetone and enter the glycolytic scheme with some of the products being utilized in virus synthesis.

The results suggested there was only a small amount of fatty acid synthesis after infection. Malonic acid, a precursor to fatty acid synthesis, was only slightly incorporated into the virus. It was of interest to note that malonic acid was incorporated into infected tissue significantly less than into control tissue. Among the long chain fatty acids there were no significant differences, with respect to C^{14} contribution to the virus, even between the saturated and unsaturated acids. All were labeled in the C-1 position, as uniformly labeled acids were not available. Although a comparison of the long chain and the short chain acids was not valid because of solubility differences, it appeared that the long chain acids were not utilized as effectively, in terms of the number of moles contributed to the virus.

The amounts of apparent incorporation from the different short chain fatty acids were significantly different only when the C_4 and C_6 acids were compared. One of these short chain acids, butyric, was unusual in that the number of molecules contributing C^{14} to the

virus was greater than for any other fatty acid and it also caused nearly a two fold stimulation in virus production. After preliminary experiments, it appeared that this stimulation was an increase in total virus production and not just a greater release of virus from the tissue. Just how the butyric acid effected the stimulation is obscure, since neither acetate nor any of the other similar short chain fatty acids caused this effect.

If it was assumed that the cholesterol-4-C^{14} was incorporated into the virus as the whole molecule, the C^{14} measured would represent a considerable amount of carbon. In order to justify this assumption, it must be pointed out that the label was in the A ring of the cholesterol molecule. It would be difficult for the host cell to convert this to another structure which could be used for virus synthesis. Even though cholesterol apparently contributed considerably to the virus, very little C^{14} was incorporated into the virus from the precursor, 3-hydroxy-3-methylglutaric acid. This would suggest that the viral cholesterol was derived mainly from preformed host material. This would be in agreement with the conclusion of Frommhagen, Knight and Freeman. They found that the cholesterol in the virus and the host tissue was present in approximately equal amounts (7, p. 185-186).

There was significant incorporation of C^{14} into the infected tissues for all of the compounds tested. In every case, although the

values were not all significantly different, the infected tissues incorporated less than the control tissues, as measured at the end of the incubation period. It has been suggested by Buckley that this might be due to the passage of the virus particle through the cell membrane causing a change in permeability (3, p. 134-136).

SUMMARY AND CONCLUSION

1. Fourteen C^{14} labeled lipids and lipid precursors were quantitatively compared for their contribution to Lee influenza virus. In addition, the incorporation of these compounds into infected and control host tissues were compared.
2. The results suggested that the long chain fatty acids contributed little to the production of new virus. The short chain fatty acids, especially butyric acid, were incorporated into the virus in significantly greater amounts than most of the other compounds tested, on a molar basis. It is quite probable that fatty acid synthesis was significantly decreased after infection. The amount of incorporation of malonic acid into infected tissues was significantly less than that incorporated into control tissues. Also, little C^{14} was found in the virus from malonic acid.
3. Butyric acid caused a significant stimulation in virus production. The explanation for this effect is unknown since acetate and other fatty acids similar to butyric did not produce this stimulation.
4. Ethanolamine and choline, found in considerable amounts in the infected tissue, did not contribute significantly to the synthesis of the virus. Glycerol however, was incorporated in moderate amounts into the virus. These data suggested the major

phosphatides of the virus, lecithin and cephalin, were preformed but that some of the minor phosphatides, such as phosphatidic acid, might have been synthesized after infection.

5. Virus cholesterol was probably mostly preformed host material. A relatively high incorporation value was obtained with cholesterol-4-C^{14}, but the precursor, 3-hydroxy-3-methylglutaric acid-3-C^{14}, contributed little C^{14} to the virus. The value for the glutaric acid derivative was one of the lowest found in both the infected tissue and the virus.
6. On a molar basis, acetate was incorporated into the virus to a greater extent than any other compound studied.
7. The amount of each compound incorporated into infected cultures was significantly less than that in control cultures for eight of the compounds tested.

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