The Calcium-Stimulated Incorporation of Ethanolamine and Serine into the Phospholipids of the Housefly *Musca domestica*

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1. The calcium-stimulated incorporation of [2-¹⁴C]ethanolamine and L-[3-¹⁴C]-serine into the phospholipids of homogenates of the fat bodies from larval houseflies (*Musca domestica*) was studied. 2. Ethanolamine and serine acted as competitive inhibitors with one another. *N*-Methylethanolamine was not distinguished from ethanolamine by the system. This buffer was also a competitor with these compounds, and a number of other amino alcohols were inhibitory, probably competitively. 3. The incorporation of [³²P]phosphorylethanolamine into phospholipids was observed in suspensions of whole fat bodies. This incorporation was stimulated by magnesium. 4. During the incubation of the homogenates, a calcium-stimulated breakdown of phospholipids by a phospholipase A occurred. 5. These results are compared with results published for similar mammalian systems, and their possible physiological significance is discussed.

A rapid incorporation of labelled serine and ethanolamine into the phospholipids of whole fat bodies of housefly larvae was observed by Crone (1967a) during an investigation of the origin of lipid ethanolamine. The origin of this ethanolamine is of special interest since phosphatidylethanolamine is the predominant phospholipid in houseflies (Crone & Bridges, 1963). The previous study showed that lipid ethanolamine originated by decarboxylation from lipid serine, and it was suggested that a cyclical scheme was in operation by which ethanolamine was produced from serine via the phosphatidyl compounds. This was an extension to insect metabolism of current ideas on mammalian phospholipid metabolism (Borkenhagen, Kennedy & Fielding, 1961). An essential feature of the proposed scheme is the exchange of ethanolamine in the phospholipid with free serine, yielding phosphatidylserine and free ethanolamine. The present paper studies the incorporation of various amino alcohols into phospholipids to assess the feasibility of such an exchange and to get some information on the reaction mechanism. It also affords a comparison of this aspect of insect phospholipid metabolism with the various calcium-stimulated exchanges reported in rat liver tissue (summarized by Hübscher, 1962).

The results are discussed in relation to the above ideas, and also to assess how far they can explain the incorporation of abnormal amino alcohols into

the phospholipids of insects (Bieber & Newburgh, 1963; Bridges, Ricketts & Cox, 1965). A preliminary report of this work has appeared (Crone, 1967b).

MATERIALS AND METHODS

Radioactive compounds. L-[3-¹⁴C]Serine (specific radioactivity 4.6 mc/m-mole) and [2-¹⁴C]ethanolamine (4.86 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Lithium aluminium hydride was used to reduce [1-¹⁴C]-sarcosine by the method of Vogl & Pöhm (1953). After destruction of the excess of reagent with water, the residue was dried and the product was collected on a cold surface after prolonged vacuum distillation. Instead of the expected *N*-methyl[1-¹⁴C]ethanolamine, a mixture of this (70%) and 30% [1-¹⁴C]ethanolamine was obtained. For the present purposes this was satisfactory, but the method cannot be recommended without modification for the preparation of the pure *N*-methylethanolamine. The specific radioactivity of the product was 0.65 mc/m-mole.

[³²P]Phosphorylethanolamine (initial specific radioactivity 100 mc/m-mole) was prepared from ethanolamine, orthophosphoric acid and [³²P]orthophosphate solution as described by Weisburger & Schneider (1961). The yield of the phosphorylethanolamine recovered free from ethanolamine was 22%.

Preparation of fat body tissue and treatment of incubations. The fat bodies were isolated from larvae and the incubations were performed as described by Crone (1967a) for whole fat bodies, except for the following points. Fat bodies were isolated under 0.9% NaCl soln. and put in a cooled glass

homogenizer tube. About 120 larvae (3.6 g.) were prepared for eight incubations. The suspension of fat bodies was centrifuged and the saline poured off. A portion (4.5 ml.) of a buffer solution (20 mM-tris or 50 mM-imidazole) was added and the tissue was homogenized by rotation and reciprocal motion of a loose-fitting pestle worked by hand. Homogenization was stopped when no visible particles remained; the liquid then resembled milk. For each incubation 0.5 ml. of this preparation was used. Either 17 mM-tris or 42 mM-imidazole buffer, with 10 mM- CaCl_2 , brought to pH 7.4 with HCl, constituted the incubation medium. All solutions were brought to pH 7.4 with NaOH or HCl before addition to the incubation medium. Final volume was 1.0 ml. Incubation time was 20 min. at 25°. These are referred to as the 'standard conditions' in the text. Any variations are noted at the appropriate place. Extraction of the lipids was performed as described previously (Crone, 1967a).

The incorporation of radioactivity in each incubation was related to the weight of whole live larvae from which the tissue was derived, to the lipid phosphorus content after incubation, and sometimes to the protein present in similar samples before incubation. The first measure is used in this paper, expressed as g. live wt. of larvae (1 g. live wt. of whole larvae $\equiv$ 2.55 μ moles of fat body lipid phosphorus $\equiv$ 7.5 mg. of fat body protein).

Thin-layer chromatography. This was done on 0.25 mm. layers of silica gel H (E. Merck A.-G.). After spreading, the plates were dried in a stream of air at room temperature. Plates for lipid separations were heated at 110° for 1 hr. just before use. Details of solvents are given in the text.

Column chromatography. For the examination of the nature of the lipid into which [$2\text{-}^{14}\text{C}$]ethanolamine was incorporated a column (internal diam. 0.6 cm.) formed from 1 g. of silicic acid (100–200 mesh) previously heated at 110° for 24 hr. was used. The column was prepared in chloroform and the sample applied in 1 ml. of the same solvent. The column was then eluted with 10 ml. portions of solvent containing increasing amounts of methanol in chloroform. The solvent composition (% of methanol in chloroform, v/v) was changed as follows: 0% (10 ml.), 2% (10 ml.), 4% (10 ml.), 10% (10 ml.), 20% (10 ml.), 40% (20 ml.) and 80% (20 ml.). Fractions of 10 ml. were collected.

Liquid-scintillation counting. Samples were prepared in a dioxan-naphthalene scintillant (Crone, 1967a) and counted in a Beckman scintillation spectrometer. Counts were corrected to a set external standard ratio to compensate for quenching. Efficiency was 90% for ^{14}C and 70% for ^{32}P .

Details of other methods will be found in Crone (1967a).

RESULTS

General factors affecting the incorporation of [$2\text{-}^{14}\text{C}$]ethanolamine into fat body lipids. The time-course of isotopic ethanolamine incorporation into lipid in homogenates in tris buffer is shown in Fig. 1. The incorporation after 20 min. was used in later experiments as a measure of initial velocity, since this point gives the greatest degree of change without an appreciable fall-off in velocity. This time of 20 min. was also used in experiments with homogenates in imidazole buffer. After 40 min. the incorporation in this buffer was 1.73 times that at 20 min., which indicates reasonable linearity at the

Fig. 1. Incorporation of [$2\text{-}^{14}\text{C}$]ethanolamine into lipid by fat body homogenates as a function of time. The tissue was prepared and incubated in tris buffer under standard conditions. Ethanolamine concentration was $124 \mu\text{M}$. The initial velocity was 1.3×10^{-8} moles/hr./g. live wt. of larvae.

shorter time. Serine and ethanolamine are metabolized by whole fat body tissue (Crone, 1967a) so that ^{14}C activity originating from them appears eventually in the glycerol and fatty acid chains of the lipid molecules. The same effect occurs in the homogenates. However, after 20 min. of incubation, the radioactivity from [$2\text{-}^{14}\text{C}$]ethanolamine was recovered from the lipids as ethanolamine only, and from L-[$3\text{-}^{14}\text{C}$]serine approx. 3% was recovered as lipid components other than serine or ethanolamine. For this short time-period therefore, the total incorporation into lipid was taken as representing incorporation into the nitrogenous bases of the phospholipids only.

The effect of pH on ethanolamine incorporation in veronal buffer is presented in Fig. 2. The optimum was between pH 7.25 and 8.25, with a sharp fall-off in activity on either side of these points. pH 7.40 was used in all other experiments reported here.

The incorporation of ethanolamine was reduced to 3% of the control value by heating the preparation in a boiling-water bath for 5 min. before adding the substrate. The incorporation of ethanolamine was actively inhibited by Mn^{2+} , Zn^{2+} and Hg^{2+} ions (Table 1). This Table also shows the

Fig. 2. Effect of pH on the incorporation of [2- ^{14}C]ethanolamine into lipid by fat body homogenates. The incubations were carried out in 75mm-veronal-HCl buffer, with 10mm-calcium chloride. Ethanolamine concentration was $63\mu\text{M}$.

Table 1. *Effects of some metal ions and surface-active agents on the incorporation of [2- ^{14}C]ethanolamine into lipids*

The experiments were performed with homogenates in imidazole buffer by the standard method. Ethanolamine concentration was 0.063mm.

Metal ion added (10mm, as chlorides)	Other additions	$10^8 \times \text{Rate of incorporation into lipids (moles/hr./g. live wt. of larvae)}$	Incorporation (percentage of control with calcium)
Ca^{2+}	None	3.30	100
Ca^{2+}	0.1% (v/v) Triton X-100	0.22	7
Ca^{2+}	Cetyl-methyl-ammonium bromide	0.09	3
Mn^{2+}	None	0.06	2
Zn^{2+}	None	0.03	1
Hg^{2+}	None	0.03	1
None	None	2.03	61.5

inhibitory effect of Triton X-100 (a non-ionic surface-active agent) and of the cationic detergent cetyltrimethylammonium bromide.

Fig. 3. Effect of calcium chloride (Δ) and magnesium chloride ($\circ$) on the incorporation into lipid of [2- ^{14}C]ethanolamine by fat body homogenates in tris buffer. Ethanolamine concentration was $63\mu\text{M}$.

A homogenate of fat body tissue was sedimented in 0.25M-sucrose. One-quarter of the enzymic activity which incorporated ethanolamine in the original homogenate was demonstrated in a fraction which sedimented after centrifuging for 5min. at 130g, and one-half of this activity was present in a second fraction which sedimented after centrifuging for 10min. at 2000g. The centrifugation yielding the second fraction would not be sufficient to sediment particles the size of mammalian mitochondria, and it is possible this fraction represents some form of vacuole in the fat body cells. This is the fraction (prepared without sucrose present) which was used to demonstrate the calcium requirement of the incorporation, and it was noticed in these experiments that much tissue was lost on repeated washing, which indicates that the particles present are fragile.

Effect of calcium and magnesium ions on the incorporation of ethanolamine and serine. The incorporation of ethanolamine radioactivity in a homogenate prepared in a buffer without metal ions was increased by the addition of Ca^{2+} ion (Fig. 3). Addition of Mg^{2+} ion caused a gradual reduction in the existing enzymic activity. A fraction was prepared from fat body tissue by homogenization in buffer containing 10mm-EDTA and sedimentation by centrifugation at 2000g for 20min. The precipitate was resuspended in buffer without EDTA and again sedimented. The ethanolamine-incorporating activity of this preparation was increased fourfold by addition of calcium to 20mm. With $63\mu\text{M}$ -ethanolamine the maximum velocity of incorporation was reached at a calcium concentration between 10 and 20mm in both 50mm-imidazole and 20mm-tris buffers. With $40\mu\text{M}$ -serine as substrate the response to calcium was markedly different in that maximum velocity was reached between 20 and 50mm-calcium in imidazole buffer.

However, when the serine concentration was raised to 1mM, maximum velocity of incorporation was observed at 10–20mM-calcium, as previously noted for ethanolamine. Thus the response to Ca^{2+} ion concentration was dependent on the substrate concentration, whereas change in buffer had no effect.

Variation of ethanolamine and serine incorporation with substrate concentration. The effect of substrate concentration on the velocity of the incorporation of the two compounds into lipid was studied in tris and in imidazole buffers with 10mM-calcium chloride. The method used was to add a constant amount of the radioactive substrate with a varied amount of the non-radioactive substrate.

Fig. 4. Relationship between L-serine concentration and the velocity of $[3\text{-}^{14}\text{C}]$ serine incorporation (Δ), and the effect of ethanolamine at 1mM ($\circ$). The experiment was performed in imidazole buffer under standard conditions.

The measured isotopic incorporation was then corrected for the diluting effect of the non-radioactive compound. A range of substrate concentration from 0.1 to 10mM was normally employed. The plots of reciprocals of velocity against reciprocal of substrate concentration in tris buffer were linear within this range. In the imidazole buffer, however, the points at 10mM were off an otherwise linear plot. As shown in Fig. 4, this loss of linearity was towards a disproportionate increase in velocity with increase in substrate concentration. This may indicate that there was either a substrate activation of the enzyme or that more than one enzyme was present. However, if these points at high substrate concentrations were ignored, a K_m value $2 \times 10^{-4}\text{M}$ for both serine and ethanolamine in imidazole buffer was calculated. The same values were obtained by measuring the incorporation of labelled serine and studying the effects of non-radioactive ethanolamine, or vice versa by using labelled ethanolamine and non-radioactive serine. The serine-ethanolamine relationship was one of competitive inhibition in both buffers, as judged by the criterion of the double-reciprocal plot (Fig. 4). As previously reported by Crone (1966), tris buffer acted as a competitor in this system, with K_i $1.8 \times 10^{-3}\text{M}$. When this factor was allowed for, the K_m values for serine and ethanolamine in tris buffer were again $2 \times 10^{-4}\text{M}$.

Results are described later which show that *N*-methylethanolamine was not distinguished from ethanolamine by the incorporating system, so that the ratio of incorporated labelled *N*-methylethanolamine to incorporated labelled ethanolamine was the same as the ratio in the medium, no matter what the actual composition of the medium was. However, the results of Table 2 show that this was not the case for the ethanolamine and serine relationship, since the isotopic ratios of the two compounds

Table 2. *Effect of variation of the medium composition on the incorporation of ethanolamine and serine*

The incubations were performed under standard conditions in imidazole buffer. Eight incubations were performed. In one set of four, $[2\text{-}^{14}\text{C}]$ ethanolamine was included (5.15×10^5 counts/min. in each incubation). In the other set of four, $[2\text{-}^{14}\text{C}]$ ethanolamine was present, but also L- $[3\text{-}^{14}\text{C}]$ serine (6.73×10^5 counts/min.) replaced an equal quantity of the non-radioactive serine. The results for radioactivity in lipid derived from $[^{14}\text{C}]$ serine (fourth column) were found by subtracting the radioactivity obtained in the first set from the radioactivity in the appropriate samples of the second set. The ratio of ethanolamine to serine radioactivity in the medium of the second set was 0.77:1.

Composition of incubation medium (mM)		Radioactivity in lipid (counts/min.)		Ratio of ethanolamine to serine radioactivity in lipid	10 ⁹ × Rate of incorporation (moles/20 min./incubation)	
		From $[^{14}\text{C}]$ -ethanolamine	From $[^{14}\text{C}]$ serine		Ethanolamine	Serine
Ethanolamine	Serine					
0.06	1.08	3440	2230	1.54	0.4	3.6
0.56	0.58	3410	1760	1.94	3.7	1.5
0.81	0.33	3260	1840	1.77	5.1	0.9
1.06	0.08	3260	2640	1.23	6.7	0.3

incorporated vary with the proportions present in the medium (compare with Table 4), and in no case approach the ratio of the two labelled compounds in the medium. The results in this Table were analysed and shown to be consistent with the previous results in the following manner. If the initial rate of incorporation (expressed as moles/20min./incubation) was plotted against substrate concentration, straight lines passing through the origin were obtained for both ethanolamine and serine. This property was due to the particular conditions of this experiment, in which the two K_m values were equal ($K_m = K_i$) and the total concentration of the two compounds (substrate and inhibitor) was constant. Under these conditions, $K_p = K_m[1 + (i/K_i)] = K_m + i$ (notation of Dixon & Webb, 1958). The Michaelis equation for competitive inhibition then became:

$$v = \frac{V}{1 + \frac{K_m + i}{s}}$$

If the total concentration of substrate and inhibitor was called 't', then

$$v = sV/(t + K_m)$$

This was the equation of the straight-line graphs obtained, whose slope was therefore $V/(t + K_m)$. From these graphs, the maximum velocity (V) was found

to be 130×10^{-9} moles/hr./g. live wt. of larvae for ethanolamine (a value of 104×10^{-9} was previously determined by a direct method) and 64×10^{-9} moles/hr./g. live wt. of larvae for serine (previous value was 63×10^{-9}).

Effects of various amino alcohols on the incorporation of ethanolamine and serine into lipids. From the results of the previous section, serine, ethanolamine and tris showed competitive effects. A survey of other amino alcohols was therefore made to see if they were similar in this respect. As reported in Table 3 a wide range of compounds showed inhibitory effects on the serine or ethanolamine incorporation. Since the experiments were done at one inhibitor concentration, it was of course impossible to decide whether the effects were due to competition or to a non-competitive inhibition. L-Serine, ethanolamine, N-methylethanolamine and N-dimethylethanolamine were indistinguishable by the inhibition they caused. D-Serine had a weak depressing effect on L-serine incorporation. A comparison of the results with 2-aminopropan-1-ol and 1-aminopropan-2-ol suggests that possession of a primary hydroxyl group enhanced the inhibition. The inhibitory effect was decreased as the amino and hydroxyl groups were moved apart (see the ω -amino-1-ol series). Increasing the carbon chain length, given the 2-amino-1-ol structure, decreased the effectiveness of the compounds as

Table 3. *Effect of various amino alcohols on the incorporation of ^{14}C radioactivity into lipid from $[2-^{14}\text{C}]$ -ethanolamine and L- $[3-^{14}\text{C}]$ serine*

Composition of the incubation media (imidazole buffer) and preparation of the tissue followed the standard procedure described in the Materials and Methods section. The experiments with $[2-^{14}\text{C}]$ ethanolamine (a concentration of 0.063 mM being used in the medium) were performed in two groups, with tissue preparations made at two different times. These are designated 'a' or 'b'. The $[3-^{14}\text{C}]$ serine experiments (0.04 mM-serine) were done with a third tissue preparation. Results are presented as the radioactivity incorporated into the lipid, expressed as a percentage of the control value obtained with no added amino alcohol. The rates of incorporation for the controls were (expressed as $10^8 \times$ moles/hr./g. live wt. of larvae): 3.2 for 'a', 3.5 for 'b' and 1.3 for the serine experiment.

Compound added to incubations	Incorporation of ^{14}C radioactivity into lipid (percentage of the control value)	
	$[2-^{14}\text{C}]$ Ethanolamine	L- $[3-^{14}\text{C}]$ Serine
10 mM-Ethanolamine	—	8
10 mM-L-Serine	4 (a)	—
10 mM-D-Serine	—	47
10 mM-N-Methylethanolamine	5 (a)	8
10 mM-N-Dimethylethanolamine	6 (a)	9
10 mM-Choline	20 (a)	23
20 mM-DL-Carnitine	92 (a)	104
20 mM-DL-2-Aminobutan-1-ol	19 (a), 14 (b)	26
20 mM-DL-1-Aminopropan-2-ol	26 (b)	—
10 mM-L-2-Aminopropan-1-ol	10 (b)	—
10 mM-3-Aminopropan-1-ol	64 (b)	—
10 mM-4-Aminobutan-1-ol	94 (b)	—
10 mM-5-Aminopentan-1-ol	96 (b)	—

inhibitors (compare the series ethanolamine, 2-aminopropan-1-ol and 2-aminobutan-1-ol). The effect of choline was much less than that of the other *N*-substituted ethanolamines, and carnitine had no effect.

Incorporation of *N*-methyl[1-¹⁴C]ethanolamine into the fat body lipids. A mixture of *N*-methyl-[1-¹⁴C]ethanolamine and [1-¹⁴C]ethanolamine was incubated with a fat body homogenate under standard conditions (imidazole buffer) in five incubation mixtures in which the concentrations of the two ethanolamines were varied by adding the non-radioactive compounds. Thus the radioisotopic content was not changed when the chemical composition was varied quite widely. Table 4 shows the distribution of the two radioactive compounds in the lipid after hydrolysis and separation by two different methods. It is obvious that the radioisotopic distribution between the lipid ethanolamines reflects that in the medium, and variation of the chemical composition has had no effect. A K_m for *N*-methylethanolamine of $2 \times 10^{-4} M$ was calculated by measuring the incorporation of [2-¹⁴C]-ethanolamine at various concentrations in the presence and absence of 1 mM-*N*-methylethanolamine. This value is similar to that previously found for ethanolamine.

Table 4. *Incorporation of a mixture of N-methyl-[1-¹⁴C]ethanolamine and [1-¹⁴C]ethanolamine into fat body lipids, and the effect of varying the chemical composition of the medium*

Incubation was performed in imidazole buffer by the standard procedure described in the Materials and Methods section. The lipid extracts were hydrolysed by HCl. Dinitrophenyl derivatives were formed from portions of the hydrolysates, and the derivatives of ethanolamine and *N*-methylethanolamine were separated by thin-layer chromatography in chloroform-benzyl alcohol-acetic acid (95:7:3, by vol.) (method 1). Other portions of the hydrolysates were separated directly on thin-layer chromatograms in methanol-water-ammonia soln. (sp.gr. 0.880)-10% trichloroacetic acid (50:30:15:5, by vol.) (method 2).

Composition of incubation medium (mM)		Ratio of <i>N</i> -methylethanolamine radioactivity to ethanolamine radioactivity in lipid	
<i>N</i> -Methyl-ethanolamine	Ethanolamine	Method 1	Method 2
0.07	0.13	2.45	2.45
0.07	0.03	2.45	2.4
1.07	0.03	2.6	2.4
5.07	0.03	2.45	2.45
10.1	0.03	2.0	2.1
Radioactivity ratio in original preparation of [¹⁴ C]ethanolamines		2.1	2.7

Nature of the lipid into which the [2-¹⁴C]ethanolamine is incorporated. Because of the reports of the formation of fatty amides of ethanolamine in various tissues (Bachur & Udenfriend, 1966) it was necessary to examine more closely the lipid extracts labelled by the [2-¹⁴C]ethanolamine. The remaining extracts from several incubation mixtures in which radioactive ethanolamine had been incorporated (to estimate K_m for this compound) were pooled, and the combined preparation was chromatographed on a column of silicic acid. Of the radioactivity applied to the column, 81% was eluted by chloroform containing 20% methanol and 40% methanol. These two fractions were each subjected to hydrolysis by mild alkali (Dawson, 1960, method I reduced to one-fifth of the original scale), and the aqueous layers were chromatographed on thin layers in ethanol-water (74:26, v/v). The areas at R_f 0.0-0.1, corresponding to marker ethanolamine, and at R_f 0.3-0.4, corresponding to glycerylphosphorylethanolamine, were scraped from the plates and assayed for radioactivity. Duplicate chromatograms were assayed for phosphorus. Of the radioactivity of these fractions 92% was recovered as glycerylphosphorylethanolamine. Similar extracts labelled by incubation with [2-¹⁴C]ethanolamine were chromatographed on thin layers in chloroform-methanol-8N-ammonia soln. (65:35:5, by vol.), after the chromatograms had been run in chloroform and dried. Radioactivity was recovered in the phosphatidylethanolamine zone, and on subsequent hydrolysis and chromatography this radioactivity behaved as glycerylphosphorylethanolamine.

The behaviour of the lipid radioactivity on column and thin-layer chromatography contrasts with that reported for fatty acid amides by Bachur & Udenfriend (1966). The water-soluble product of alkaline hydrolysis is glycerylphosphorylethanolamine, not ethanolamine which one would expect as the product from an *O*-acyl ester of ethanolamine. There is therefore no doubt that the ethanolamine radioactivity is being incorporated into a phospholipid.

Incorporation of [³²P]phosphorylethanolamine into the lipids of whole fat bodies. This study was undertaken to afford a comparison with the calcium-stimulated incorporation system. Whole fat bodies were prepared and incubated in 42 mM-imidazole-hydrochloric acid buffer, pH 7.4, for 1 hr. at 25° with 53 μM-[³²P]phosphorylethanolamine. The incorporation of the phosphorylethanolamine into the lipids was stimulated by magnesium rather than calcium. Cytidine 5'-triphosphate in the presence of magnesium had a weak stimulatory effect whereas the addition of phosphorylethanolamine produced a marked increase in incorporation. The incorporation was very dependent on the concentra-

tion of fat body tissue in the incubation mixture, indicating a requirement for many cofactors. These results were similar to those obtained for the incorporation of [^{32}P]phosphorylcholine into whole fat bodies of the blowfly *Sarcophaga* (Crone, Newburgh & Mezei, 1966). 73.5% of the radioactivity in the lipids after incubation behaved as phosphatidylethanolamine when the lipids were separated on thin layers of silicic acid with chloroform-methanol-8N-ammonia soln. (65:35:5, by vol.) as solvent. In the aqueous extract of the incubation mixture, 30% of the radioactivity behaved as cytidine diphosphorylethanolamine when the extract was chromatographed on thin layers of silicic acid with methanol-water-ammonia soln. (sp.gr. 0.880)-10% trichloroacetic acid (50:30:15:5, by vol.) as solvent. Although the latter identification was by no means definite, the whole pattern of these results suggested that the fat bodies contained a system incorporating phosphorylethanolamine that was similar to that demonstrated by Kennedy and Weiss (1956) in rat liver.

Breakdown of fat body phospholipids during incubation of the homogenates. The disappearance of total lipid phosphorus and of phosphatidylethanolamine phosphorus during incubation in buffer containing either 10mM-calcium or 8mM-EDTA was compared. The phosphatidylethanolamine was isolated by thin-layer chromatography in chloroform-methanol-8N-ammonia soln. (65:35:5, by vol.). When the cation concentration was low there was very little loss of phosphorus from the lipids as a whole. However, the presence of calcium caused a fall in the phosphorus content both in the total lipid and in the separated phosphatidylethanolamine. The loss of phosphorus from the separated lipid averaged 90% of the loss from the total lipid, and since the phosphatidylethanolamine content of these fat bodies was determined as 48% of the total lipid phosphorus, this must mean that the calcium-induced degradation affected this lipid more than it did the other phospholipids present. The extracts from which these results were obtained were also examined for phosphatidic acid content by chromatography on small silicic acid columns in chloroform-methanol (95:5, v/v) (Crone, 1964). These results were somewhat variable, but showed no trend towards an increase with time, nor any difference between the calcium and EDTA incubation values, save that the EDTA values were always slightly higher. The mean of the figures for the calcium buffer incubations was 4.7% of the initial lipid phosphorus, and for the EDTA buffer incubations 6.1%.

The degradation of the homogenate lipids in buffer containing 10mM-calcium was also followed in a preparation which had previously been in-

cubated with [^{32}P]phosphorylethanolamine. A marked rise occurred in the lysophosphatidylethanolamine fraction which reached 14% of the initial phosphatidylethanolamine radioactivity after 2hr. This rise at first exceeded the loss of radioactivity from the lipids as a whole, but after 1hr. the position was reversed. These results are consistent with a calcium-stimulated breakdown of phosphatidylethanolamine via the lyso compound, mediated by a phospholipase A.

There was no doubt that during the study of the calcium-stimulated exchange reaction the phospholipids were breaking down, but the change after 20min., the normal incubation period, was not greater than 5%.

Incorporation of [^{14}C]ethanolamine by pig liver microsomes. The effects of various factors on the incorporation of ethanolamine into lipid by pig liver microsomes are presented in Table 5. This experiment was performed to give a general comparison with the results for the housefly (compare with Table 3). The incorporation was depressed by other amino alcohols, including tris, but the effect was less marked than in Table 3. The control rate of incorporation, expressed relative to lipid phosphorus content, was very much lower than in the housefly preparations (4×10^{-10} moles/hr./ μmole of lipid phosphorus compared with an average value of 1.3×10^{-8} for the housefly). The mammalian system may be far from its optimum

Table 5. *Incorporation of [^{14}C]ethanolamine by pig liver microsomes and the effect of some amino alcohols*

The incubation medium consisted of 0.1 ml. of microsome suspension in 1.15% (w/v) KCl (Brookes & Harrison, 1966) and various additions as shown below in 37mM-imidazole buffer with 10mM-calcium chloride, pH 7.4 (in the first incubation the calcium chloride was omitted). Ethanolamine concentration was 63 μM . Final volume was 1.0 ml. The incubations were carried out at 25° for 20 min. Other experimental details were as described for the housefly preparations. Results are expressed as a percentage of the incorporation by the control incubation with 10mM-calcium and no other additions. The control rate of incorporation was 4×10^{-10} moles/hr./ μmole of lipid phosphorus.

Addition to incubation medium	^{14}C radioactivity incorporated (percentage of the control)
None (no calcium)	32
None	100
10mM-L-Serine	15
10mM-Tris	80
10mM-N-Methylethanolamine	28
10mM-L-2-Aminopropan-1-ol	21
10mM-3-Aminopropan-1-ol	88

temperature at 25°, however. These results show that a mammalian preparation gives broadly similar results to housefly preparations under the particular conditions used here.

DISCUSSION

This paper describes a calcium-stimulated system in housefly fat body homogenates which incorporates labelled ethanolamine and serine into phospholipids. It is proposed to discuss these results first in relation to the similar systems found in mammalian tissue, and secondly with regard to their significance in insect physiology.

The incorporation of nitrogenous bases into phospholipids has been shown to follow two distinct patterns in mammalian tissue. A method of net synthesis dependent on cytidine nucleotides and magnesium has been demonstrated by Kennedy & Weiss (1956). In contrast with this, a number of observations have been made of calcium-stimulated incorporations which are generally regarded as exchanges not requiring energy. These observations include those of Artom (1961) on ethanolamine incorporation, of Dils & Hübscher (1961) on choline incorporation, of Hübscher (1962) on serine incorporation and of Borkenhagen *et al.* (1961) on the incorporation of both ethanolamine and serine. For various reasons the calcium-stimulated incorporations observed in mammalian tissue have been assigned to different enzymic systems, whereas the present results for housefly tissue show no such division. It is the author's opinion, however, that many of the apparent differences between the mammalian systems are not real when closely examined. Thus the ethanolamine incorporation observed by Artom (1961) was originally described as requiring ATP, but C. Artom (personal communication) now believes that this could be an indirect effect related to calcium uptake. Therefore in the mammalian systems and the housefly no calcium-stimulated incorporation requiring ATP has been confirmed. A second point of difference, this time between serine and choline incorporation, was advanced by Hübscher (1962) when he compared the optimum calcium concentrations for incorporation of these two bases. A similar apparent difference was found in the housefly between the responses of the ethanolamine and serine incorporations to calcium ion concentration. However, the present author associates this with differences between the affinities of the amino alcohols for calcium, rather than to differences in possible enzymes. Further, the response to calcium ion concentration in the housefly appears to be also dependent on the absolute concentration of the substrate, so that there is a three-part relationship between enzymic activity, calcium concentration

and substrate concentration. The simplest mechanism to explain these results would involve the formation of an amino alcohol-calcium complex, which is then the immediate substrate of the enzyme. The formation of such complexes between metals and tris or ethanolamine has been discussed by Mahler (1961). Thus a difference in response to calcium concentration is not a good criterion for the existence of different enzymes. Similar considerations will apply to differences in response to pH. Hübscher (1962) gives a pH optimum of 8.1 for serine incorporation and Dils & Hübscher (1961) a value of 9.0 for choline incorporation, whereas the present results show ethanolamine incorporation to be optimum around 7.7 for housefly tissue.

A further degree of unity in the mammalian systems is shown by the observation of Borkenhagen *et al.* (1961) that there is direct competition between ethanolamine and serine, and by Dils & Hübscher (1961) who found competition between choline and L-serine and D-serine. In the housefly, competition was demonstrated between ethanolamine, serine, N-methylethanolamine and tris buffer. Participation of many other amino alcohols was indirectly indicated. The results for the housefly are therefore thoroughly consistent with the existence of a single enzyme mediating these various changes.

The present results regarding the effect of magnesium on the incorporation in housefly tissue are very similar to those of Dils & Hübscher (1961), and, as these authors imply, there might be competition between magnesium and calcium, either for the substrate or for the enzyme. Heavy metal ions have the same inhibitory effect as that reported by Hübscher (1962).

The comparison between the present results with housefly tissue and those reported for rat liver therefore show a great deal of similarity. This comparison would be much clearer if the various reported differences in the rat liver tissue could be reconciled. In connexion with this, it is relevant to state that the workers on rat liver tissue used tris buffer (Artom does not state what buffer was used). If tris is a competitor in the rat liver system as it has been shown to be in the housefly tissue (Crone, 1966), then the results obtained in different concentrations of this buffer will not be strictly comparable. The results given here for the pig liver microsomes show that the results obtained for the housefly can be compared quite validly with this mammalian system, under similar conditions of investigation.

It seems clear from the present results with labelled phosphorylethanolamine and previous results with phosphorylcholine (Crone *et al.* 1966) that the cytidine nucleotide-magnesium pathway for incorporation of amino alcohols also exists in insect fat body tissue.

The present study of phospholipid degradation is very similar to that reported by Bjørnstad (1966) to occur in rat liver microsomes. In both cases the degradation is stimulated by calcium and favours the breakdown of phosphatidylethanolamine over that of other phospholipids. The degradations both proceed through lysophosphatidylethanolamine as an intermediate, presumably by the successive action of phospholipase A and a lysophospholipase. These results are interesting in view of the suggestion by Dils & Hübscher (1961) that the calcium-stimulated incorporations may be due to a reversal of phospholipase D (phosphatidylcholine phosphatidohydrolase; EC 3.1.4.4). In this case phosphatidic acid would be expected to be an intermediate in the calcium-stimulated degradation. The methods used in the study on housefly tissue were not sensitive enough to confirm or deny the accumulation of phosphatidic acid, except that within wide limits (comparable with the accumulation of lysophosphatidylethanolamine) no change was found on incubation. This suggestion of phospholipase D participation must therefore remain an open question. Certainly an alternative route of phospholipid breakdown is present and operating in the phospholipase A system although such a system could not be involved in the exchange of free and phospholipid-bound amino alcohol. The reversal of a phospholipase D would be a more tenable explanation of the incorporation observed in the present paper but such an enzyme has never been reported to occur in animal tissue.

To discuss the physiological significance of the calcium-stimulated exchange, it is first necessary to decide what such a system could do. The simplest mechanism consistent with the results is to postulate that the phospholipids are in equilibrium with the free amino alcohols through an enzyme-phosphatide complex, as in the scheme below.

It would then be the reverse of this process, as shown here, which has been studied and has been found to be calcium-stimulated and to show competitive effects with various amino alcohols. Introduction of a labelled amino alcohol into such a system would result in the label appearing in the phospholipids. If each phospholipid is in equilibrium with the appropriate amino alcohol through an enzyme system which is specific to that amino alcohol, then no chemical change in phospholipid composition could occur. However, if the enzyme system is common to several phospholipids and the respective amino alcohols (through a common phosphatidyl-enzyme complex), then a change in relative concentrations or in the chemical nature of

the free amino alcohols would result in a change in composition of the phospholipids. The actual change would be a result of several factors, e.g. the relative concentrations of the amino alcohols and phospholipids available to the enzyme system, and the affinities of these compounds for the enzymes. It is only in this case of a common system by which the phospholipid composition is changed that a metabolic function can be ascribed to the process.

In the present experiments labelling by ethanolamine, serine and *N*-methylethanolamine has been shown to occur, and a number of other compounds have been shown to compete with this labelling. Competition has been shown to be equivalent with incorporation in the three cases of the isotopic compounds, but whether this is true for the other compounds is not known. Thus the effect of tris, for instance, might be confined to competition at the enzyme surface, or tris might actually be incorporated into phospholipid, excluding the more normal bases. Actual incorporation of a base not normally present in the phospholipids has been observed only with *N*-methylethanolamine, and it is possible to argue that this is a special case anyway, because to the incorporation system it is effectively ethanolamine itself. However, this similarity extends, to a lesser degree, to all the bases which might have a physiological role in such a system, e.g. serine, ethanolamine and the two *N*-methyl-ethanolamines. Thus the exchange of one base in a phospholipid molecule for a chemically distinct one has been shown to occur for one compound (*N*-methylethanolamine) and it is probable that this can be extended to all the normal phospholipid bases.

The present study is a continuation of previous work (Crone, 1967*a*) on the origin of phospholipid ethanolamine. Borkenhagen *et al.* (1961) postulated that phosphatidylethanolamine is formed by decarboxylation of phosphatidylserine and that the ethanolamine is released from the lipid by exchange with free serine so that a cyclical system operates. Such a release may well be important *in vivo* to supply the ethanolamine required for new synthesis of phosphatidylethanolamine. The key question to be answered before such a cycle can be considered to operate is whether free serine will displace ethanolamine from the phospholipid. Both the results of Borkenhagen *et al.* (1961) and the present results show that this is likely but do not give direct confirmation.

Work on the blowfly *Phormia regina* (Bieber & Newburgh, 1963) has shown that replacement of dietary choline with β -methylecholine, *N*-dimethylethanolamine or *N*-dimethylisopropanolamine results in the replacement of the phospholipid choline with the added base. Similar results have been obtained by Bridges *et al.* (1965) in the

housefly *Musca domestica*, with other bases. Bridges & Ricketts (1967) have extended these observations to a wide range of amino alcohols, with which replacement also occurs, to a lesser degree, when choline is present. Comparison of their results with the effectiveness of these compounds as competitors in the present study (Table 3) shows a broad correlation between degree of incorporation *in vivo* and competition *in vitro*. The most marked exception concerns *N*-dimethylethanolamine, which is not much less effective as a competitor than *N*-methylethanolamine, but is incorporated *in vivo* to a much smaller degree. It is therefore possible to explain the replacement of the bases *in vivo* as being a result of the calcium-stimulated exchange. Net synthesis of the normal phospholipids by the cytidine nucleotide pathway could be followed by an exchange with other bases present, so that the final phospholipid composition would depend on both the net synthesis and the amino alcohols available for exchange. Further work is necessary to confirm this relationship between the results obtained *in vitro* and the changes observed *in vivo*.

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