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Further Investigations of the Incorporation of [1-^{14}C]Acetate into the Lipids of the Silkworm *Bombyx mori* L.

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1. After the injection of sodium [1-^{14}C]acetate, the highest incorporation of ^{14}C into the lipids of the silkworm was observed after 24hr. 2. The specific radioactivity of the palmitic acid fraction was greater and increased more rapidly than that of the stearic acid fraction, which was consistent with the precursor–product relationship to be expected on the basis of current concepts of fatty acid synthesis *in vivo*. 3. The results indicate the probability of synthesis of lipid components in tissues other than the fat body. 4. Fractionation studies indicate considerable differences in the rate of incorporation of [1-^{14}C]acetate into neutral lipids and phospholipids between larvae and pupae as well as among tissues of larvae. 5. The rate of incorporation of [1-^{14}C]acetate remains constant throughout pupal development.

In a previous paper (Sridhara & Bhat, 1964) it was shown that the incorporation of [1-^{14}C]acetate followed a pattern similar to that observed in other insect species; for example, little or no incorporation was found in the C_{16}–C_{18} polyunsaturated fatty acids of the saponifiable lipids. From the reports of Phil & Bloch (1950) and Van Bruggen, Hutchens, Claycomb & West (1953) it was clear that in a study of this kind importance should be given not only to the time-course of labelling of the lipid in the intact animal, but also to the rates at which the fatty acids enter into ester linkages in different organs and different lipid components. With these in view a detailed study was undertaken with the silkworm, and the results recorded are presented in this paper.

MATERIALS AND METHODS

Mature larvae of *Bombyx mori* L. were injected with [1-^{14}C]acetate solution (1·4mc/m-mole). The method of injection and the procedures for extracting and separating the labelled lipids were as detailed by Sridhara & Bhat (1964). For comparing the incorporation of ^{14}C into the lipids of different tissues, a number of larvae were given [1-^{14}C]acetate and kept for 8hr. at room temperature (23–25°). They were dissected in the cold, and the organs were removed, washed with water and pooled separately. Some larvae injected with [1-^{14}C]acetate were allowed to spin cocoons, and the 5-day-old pupae therefrom were taken for analysis; this is referred to below as the 'pupa-∞' sample. Pupae from the beginning of pupation to the moth stage were injected with [1-^{14}C]acetate on alternate days.

The lipids were fractionated on silicic acid by two procedures, namely those of Hirsch & Ahrens (1958) and Barron & Hanahan (1958), after standardizing the steps for eluting the respective components by the eluents mentioned in Table 3, a mixture of authentic compounds being used for this purpose.

RESULTS

Table 1 shows that the specific radioactivity of the silkworm lipids increased during the first 24hr., the maximal specific radioactivity being attained between 24 and 48hr. The highest radioactivity in the saponifiable fraction was recovered in palmitic acid, notwithstanding the fact that the percentage incorporation into the oleic acid fraction increased with time. The total radioactivity (15%) incorporated in the larvae was lost, reaching a low value (4%) in the pupa-∞ sample. Also, the percentage of the radioactivity within the components of the saponifiable fraction changed during metamorphosis, with the simultaneous transference of the maximal amounts of radioactivity from palmitic acid to oleic acid.

Table 2 shows the distribution among various tissues of the insect of the total ^{14}C incorporated into larvae, together with the relative distribution of radioactivity between the unsaponifiable and saponifiable fractions. The maximum incorporation occurred in the fat body and the least in the intestines, though the radioactivity from the unsaponifiable fraction of the fat body was negligible compared with the high radioactivity recorded for the silk glands. High radioactivity in the saponifiable fractions of the silk glands and the intestines was recovered in stearic acid, whereas that of the fat body resided mostly in palmitic acid. In the

Table 1. *Rate of incorporation of* [1-^{14}C]*acetate into silkworm lipids*

Experimental details are given in the text.

Time after injection (hr.)	Radioactivity administered per larva (μc)	Incorporation of radioactivity (%)	Percentage of radioactivity of lipid		Radioactivity in fatty acids					
					Palmitic acid		Stearic acid		Oleic acid	
			Saponifiable fraction	Unsaponifiable fraction	Percentage of counts in saponifiable fraction	Sp. radioactivity (counts/min./mg.)	Percentage of counts in saponifiable fraction	Sp. radioactivity (counts/min./mg.)	Percentage of counts in saponifiable fraction	Sp. radioactivity (counts/min./mg.)
1	1·0	4·4	54·4	10·9	65·5	154	16·4	49	11·0	19
2	1·0	6·8	55·8	12·5	60·0	185	21·2	59	14·4	34
4	1·0	8·2	56·6	14·8	55·4	236	23·1	110	16·3	52
6	1·0	9·9	61·0	18·2	51·0	262	25·5	147	17·9	69
8	1·0	11·2	63·0	19·5	48·5	291	25·4	170	18·6	78
12	1·0	13·5	63·8	22·4	48·3	349	26·8	217	19·0	103
24	1·0	15·3	65·8	22·0	48·7	450	26·5	357	19·8	138
48	1·0	12·0	67·9	23·3	43·1	298	24·4	189	24·4	136
∞	1·0	3·9	71·0	13·8	28·3	105	13·3	88	51·0	92

Table 2. *Incorporation of* [1-^{14}C]*acetate into lipids of silkworm tissues*

Fifteen silkworms were each administered with 1·0 μc of [1-^{14}C]acetate. They were allowed to feed on mulberry leaves for 8 hr. They were dissected in the cold and organs were removed and pooled for lipid extraction. In a control batch the total incorporation was 10·5%.

Tissue	Percentage of radioactivity incorporated by the whole larval lipid	Percentage of radioactivity incorporated into lipid		Radioactivity in fatty acids					
				Palmitic acid		Stearic acid		Oleic acid	
		Saponifiable fraction	Unsaponifiable fraction	Percentage of counts in saponifiable fraction	Sp. radioactivity (counts/min./mg.)	Percentage of counts in saponifiable fraction	Sp. radioactivity (counts/min./mg.)	Percentage of counts in saponifiable fraction	Sp. radioactivity (counts/min./mg.)
Intestines	6·0	54·0	19·0	19·9	42	65·4	93	5·8	10
Fat body	66·0	86·0	2·0	67·8	405	8·3	212	15·3	114
Silk glands	9·0	42·0	20·0	36·5	140	48·2	61	14·6	20
Integuments	12·0	50·0	16·0	30·2	134	26·2	145	41·2	93

Table 3. *Fractionation of lipids from larvae and pupae of the silkworm given* [1-^{14}C]*acetate 8hr. previously*

	Percentage of radioactivity eluted						
(*A*) Method of Hirsch & Ahrens (1958)							
Eluent	Petroleum	1% Ether	6% Ether	10% Ether	25% Ether	Ether	Methanol
Fraction	Hydrocarbons	Sterol esters	Triglycerides + fatty acids	Sterols	Diglycerides	Monoglycerides	Phospholipids
Larva	7·3	3·7	62·1	2·4	4·6	2·6	11·9
Pupa	6·0	3·0	8·0	0·0	3·0	36·0	40·0
Pupa-∞	4·0	3·0	39·0	1·0	16·4	11·6	20·3
(*B*) Method of Barron & Hanahan (1958)							
Eluent	Hexane	15% Benzene in hexane	7% Ether in hexane	15% Ether in hexane	30% Ether in hexane	Ether	Methanol
Fraction	Hydrocarbons	Sterol esters	Triglycerides + fatty acids	Sterols	Diglycerides	Monoglycerides	Phospholipids
Larva	7·0	3·0	64·0	2·0	5·0	3·0	12·0
Pupa	7·0	3·0	9·0	4·0	3·0	34·0	38·0
Pupa-∞	5·2	2·4	35·6	1·0	14·0	11·4	22·0

Table 4. *Fractionation of the lipids of the tissues of the silkworm given* [1-^{14}C]*acetate 8hr. previously*

	Percentage of radioactivity eluted						
Eluent	Petroleum	1% Ether	6% Ether	10% Ether	25% Ether	Ether	Methanol
Fraction	Hydrocarbons	Sterol esters	Triglycerides + fatty acids	Sterols	Diglycerides	Monoglycerides	Phospholipids
Tissues							
Intestines	5·3	1·4	29·8	2·5	3·9	5·3	44·5
Fat body	4·4	3·6	60·5	0·4	8·7	6·4	8·5
Silk glands	6·4	2·2	41·8	3·4	1·8	3·0	32·3
Integuments	3·8	1·2	51·6	1·9	2·2	4·5	31·5

Table 5. *Incorporation of* [1-^{14}C]*acetate into silkworm lipids during pupal development*

Time after start of spinning (days)	Radioactivity administered per pupa (μc)	Percentage of radioactivity incorporated into the total lipid	Percentage of radioactivity incorporated	
			Saponifiable fraction	Unsaponifiable fraction
2	1·0	5·5	55	18
4	1·0	5·3	59	21
6	1·0	5·6	56	20
8	1·0	5·6	60	15
10	1·0	5·5	55	21

integuments, oleic acid carried the maximum radioactivity.

In Table 3 are the results of the fractionation of larval and pupal lipids on silicic acid by the two methods indicated. In keeping with the previous results, the radioactivity recovered in the sterol fraction was negligible. The separation of neutral lipids from phospholipids by both fractionation systems revealed differences between the larvae and pupae, in that the percentage of ^{14}C that entered into pupal phospholipids was more than three times that into those of the larvae. A surprising difference between larva and pupa was the high proportion (70%) of the radioactivity present in monoglycerides in the pupa. The fractionation of the lipid from the pupa-∞ sample gave evidence to the existence of an intermediate stage between that of larval and pupal lipids, in that the radioactivity

in the phospholipid and glyceride fractions increased compared with larval lipids, and that of monoglycerides decreased compared with pupal lipids.

Table 4 shows the distribution of radioactivity among different fractions of tissue lipids. Minimum incorporation occurred into the phospholipid fraction of fat body. All the tissue lipids showed negligible radioactivity in the sterol fraction, and the radioactivity found was due to contamination by non-sterol ^{14}C, since digitonides prepared from these fractions did not show any radioactivity.

The rate of incorporation of [1-^{14}C]acetate into pupal lipids during alternate days of pupal development is shown in Table 5. There was only a negligible difference in incorporation.

DISCUSSION

There appears to be a vital difference in the rate of incorporation and retention of radioactivity in the mammal and the insect, as represented by the rat on the one hand and the silkworm on the other. Whereas in the rat maximum incorporation occurred within 30min. of the administration of acetate and its radioactivity remained constant for 480min. (Van Bruggen *et al.* 1953), in the silkworm the amount of radioactivity incorporated increased up to 24hr. and then a loss of radioactivity commenced. From the percentage of radioactivity, or even the specific radioactivity of the individual fatty acids, it is evident that palmitic acid is the fatty acid that is synthesized and accumulated first by this insect, and the gradual increase in the radioactivity of stearic acid and oleic acid subsequently are pointers to the possibility of the existence in this insect also of the usual route of their synthesis, namely elongation of the palmitic acid chain and dehydrogenation of stearic acid.

A general observation that emerges from the results is that the fat body is a site of synthesis of lipids in the silkworm, and this is in conformity with experiments *in vitro* made on cell-free systems of the fat body of other insects (Zebe & McShan, 1959; Teitz, 1961). On the basis of counts/min./mg. of fat in each organ, the fat body, intestines, silk glands and integuments have radioactivity in the relative proportions 100:47:78:49. Popják & Beeckmans (1950) argued that the presence of higher radioactivity in the phospholipids of intestines than in those of liver indicated extrahepatic formation. If so, it can be argued that in the silkworm the fat body is not the only site for lipid synthesis.

The fact that the maximum incorporation of radioactivity in the saponifiable fraction of lipids from intestines and silk glands occurs into stearic acid and that of fat body into palmitic acid may have a bearing on the preponderance of phospholipids in the former two tissues, for which, in turn, the preferred saturated fatty acid is stearic acid (S. Sridhara & J. V. Bhat, unpublished work). The high rate of incorporation of ^{14}C into the silk-gland phospholipids may indicate some relation to their function in protein secretion, as suggested by Hunter & Godson (1961, 1962).

Much of the information on the distribution of incorporated radioactivity from [1-^{14}C]acetate between neutral lipids and phospholipids in the rat was obtained by the acetone-precipitation (of phospholipids) method, which Phil & Bloch (1950) themselves had stated to be not completely dependable. In fact, this procedure was found to result in a loss of more than 30% of the phospholipids in the acetone-soluble portion in the silkworm lipid (S. Sridhara & J. V. Bhat, unpublished work). Since during larval triglyceride synthesis the monoglyceride fraction does not carry much radioactivity, whereas it does during pupal phospholipid synthesis, it may be argued that monoglyceride is an intermediate for phospholipid synthesis. The fact that during the increase in the radioactivity of phospholipids during metamorphosis both the mono- and di-glyceride fractions acquire considerable radioactivity lends support to the above conclusion. Partial evidence in favour of this hypothesis may be found in the observation by Chino & Gilbert (1964), who showed diglycerides to be the transport form for fatty acids in the silkworm pupae.

The fractionation of the lipids from individual organs indicates that the radioactivity was primarily located in the phospholipids of those organs wherein the phospholipids are the major constituents and in the neutral lipids of the fat body. However, the mono- and di-glyceride fractions of fat body alone carry some radioactivity. The fact that the fat body is most probably the site of synthesis of fatty acids leads to the conclusion that the mono- and di-glycerides are essential intermediates in triglyceride synthesis. If so, there should be some mechanism that controls the entry of monoglycerides into triglycerides mainly during larval life and into phospholipids mainly during pupal life. This consideration gains importance when it is reckoned that, though the intestines and silk glands undergo histolysis and resorption during metamorphosis and considerable transformation occurs in the integuments, the fat body alone stays as such and continues in action throughout the pupal life.

Work on the incorporation of [1-^{14}C]glycine into the lipids of the silkworm (S. Sridhara & J. V. Bhat, unpublished work) had shown the appearance of two peaks during pupal life. However, no such

variation was observed at the time of incorporation of [1-^{14}C]acetate into lipids during pupal life.

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