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THE EXCRETORY ROLE OF PTERIDINES IN INSECTS

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INTRODUCTION

In 1895, Hopkins described the white and yellow wing pigments of Pieridae as '...excretory substances which function in ornament...' (Hopkins, 1895). In those days, it was generally recognized that purines, especially uric acid, were excretory products. Hopkins thought that the white substance was uric acid and that the yellow one was a derivative. In 1925 Wigglesworth still believed the wing pigments of Pieridae to be uric acid and uric acid derivatives. He based a quantitative study on this assumption, and compared storage and excretion of uric acid in *Pieris* and *Vanessa*. He agreed with Hopkins's opinion that the white pigment was an excretory substance used in ornament (Wigglesorth, 1925).

The whole basis for this opinion crumbled in the next year when Schöpf and Wieland published their discovery that the white wing pigment was not uric acid but leucopterin, a substance closely resembling uric acid, but belonging to a different chemical group (Schöpf & Wieland, 1926).

In 1933 the same workers compared leucopterin and uric acid levels in *Pieris*. They found no evidence that the two substances are metabolically connected. There is leucopterin and no uric acid in the wings; there is uric acid and no leucopterin in the excreta (Wieland, Metzger, Schöpf & Bülow, 1933). Becker took up the 'storage excretion' problem next. He did not consider pteridines excretory products (Becker, 1937*a*) and published a special paper on the subject (Becker, 1937*b*), basing his rejection of the storage excretion theory on the examination of the meconium of a series of insects all known to contain high amounts of pteridine in the adult. He failed to find any pteridines in the meconium, although he did find uric acid. It is strange that Becker's work was generally accepted without much criticism as he does not seem to have looked at the meconium of those insects which do not contain pteridines in the adult stage. If the presence of pteridines in the adult was a case of storage excretion, then one would expect true excretion in insects lacking pteridines. Since Becker's 1937 publications, it has generally been assumed that pteridines are not excretory products, although it has also been recognized that the wing pigments of Pieridae contain large amounts of highly oxidized, immobilized nitrogen.

Furthermore, pteridines are found in trace quantities in any excreta which have been examined closely. Koschara's work in 1936 is the first instance of a pteridine being recognized as an excretory substance. He reported the isolation of the pteridine uropterin (later to be recognized as xanthopterin) from human urine (Koschara, 1936), and described it as a minor excretion of a highly specialized substance, rather than as

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an end-product of nitrogen metabolism (Koschara & Haug, 1939). This opinion was strengthened with the discovery that xanthopterin excretion increases when folic acid dietary intake is increased (Rauen & Haller, 1950).

In contrast to the vertebrate situation, some insect species excrete relatively large amounts of pteridine (Berridge, personal communication). Excretion of pteridines in insects has barely been studied so far. Only very small amounts are excreted by *Pieris brassicae* (Harmsen, 1966*a*), but in *Drosophila* there appears to be a considerable pteridine excretion (Kürsteiner, 1961). In view of the limited approach of Becker (1937*a*, *b*) and the recent findings of the writer (Harmsen, 1963; 1966*b*), it appeared worthwhile to re-examine the position of the simple pteridines in the nitrogen economy of the insect.

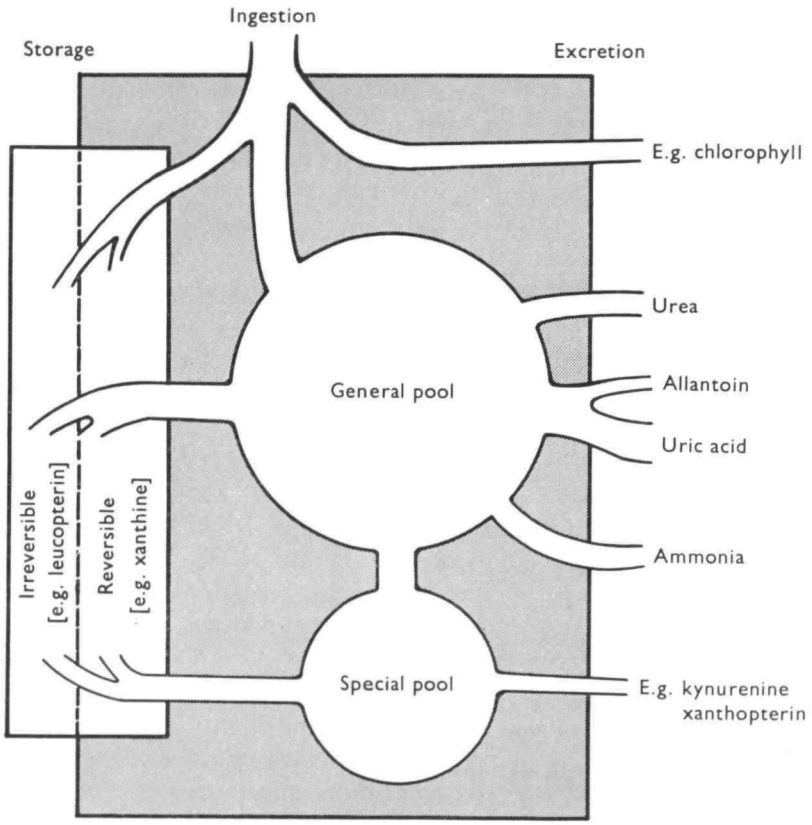

Fig. 1. Diagrammatic representation of nitrogen pathways in an insect.

Any nitrogen-containing substance, or series of substances, found in the body of a living organism or in its excreta, falls into one of the categories of the simplified diagram represented in Fig. 1.

Some ingested nitrogenous substances are excreted either directly without absorption into the body, or immediately after absorption. In this category fall such substances as chlorophyll when ingested by caterpillars. All other substances enter the general pool; thus they come within the sphere of the enzyme systems of the body and, either in the original form or after biochemical conversion, can play a part in the

functioning of the organism or merely be stored for possible future use. In its simplest form, this scheme assumes that the movement within the body from the pool to storage is a freely reversible process, and that all nitrogen-containing substances within the pool are interchangeable. In fact this is not so. Many, usually more complicated, substances leave the potential pool despite the fact that they remain within the body, either because movement is physiologically blocked or because biochemical change is impossible (i.e. the lack of enzyme systems), harmful or merely disadvantageous. These substances could be considered as being in a specialized pool. Others, particularly the more highly oxidized ones, will be excreted.

Excretion can take place directly or after the substance has been transformed into a suitable form with which the excretory mechanism can cope. The usual chemical forms of nitrogenous waste products in insects are uric acid and allantoin, and to a lesser degree urea and ammonia. However, many substances are excreted more or less directly from the special pool. These substances, especially when of a high N+O/C+H ratio (e.g. purines and kynurenine), would need to undergo a considerable biochemical change at the expenditure of metabolic energy to become standard excretory products. It is therefore not surprising to find these specialized substances either unchanged or in a slightly more oxidized form in the excreta.

Cutaneous or fat body deposits of non-functional, nitrogen-containing substance (especially in animals with relatively long lives) can be considered as storage of specialized substances which could re-enter the pool during periods of starvation or unbalanced nutrition. Any substances in the wings of Lepidoptera, however, are permanently excluded from the living body and could be considered excreted. Since many lepidopterous pupae live for a considerable time under conditions in which water uptake is impossible, and since most of these pupae will produce a large amount of nitrogen-containing waste during imaginal development, it would be advantageous to such an animal to possess a system of dry excretion of substances with a high N+O/C+H ratio. The deposition of such substances in the cuticular scales of the pharate adult could be exactly such a system. On the other hand, it would be folly to consider every nitrogen-containing cuticular pigment a form of excretion.

The possibility cannot *a priori* be excluded that pteridines, when encountered in small quantities in the body or in the excreta, originate with the ingested food and thus are not part of the nitrogen pool. Another possibility which must be taken into account is that the pteridines are synthesized by microbial commensals in the gut or fatbody. However, the hypothesis that insects are capable of synthesizing pteridines from general metabolic intermediates is much more likely (Harmsen, 1966*b*).

Left to be settled is whether the pteridines are part of the general pool or part of a specialized pool. This distinction is of little importance as far as functional substances are concerned. Pteridines of the folic acid series, eye pigments, etc., must be considered functional. The distinction between general pool and specialized pool, however, becomes significant when excreted or stored pteridines are considered. When small quantities of pteridines are found in the excreta, they can be classified confidently as belonging to excretory end-products from a specialized pool, especially when the amounts excreted can be accounted for by the presence of comparable amounts of functional pteridines in the living organism. However, when quantities of pteridines, comprising a sizeable fraction of the total excreted nitrogen, are found in

the excreta, and when only a small part of these excreted pteridines can be accounted for as being end-products of functional substances, there is a distinct possibility that pteridines are synthesized in the general pool merely as an end-product of general nitrogen metabolism. In this case, the pteridines will have to be considered on the same level as the better-known excretory products such as uric acid, urea and ammonia.

The problem of storage is complicated by the fact that even an irreversibly stored substance can perform a function, e.g. pigmentation. On the other hand, pigments can perform the function of storage excretion. The differentiation between these two views takes on considerable significance only when considered on an evolutionary basis. Originally, pteridines must have been either synthesized as wing pigments or deposited in the cuticular scales as a form of excretion; both these alternatives may have had evolutionary advantages. In the first instance, the pigments, being highly oxidized, nitrogen-containing substances, would have automatically performed an excretory role as well. In the second instance, the excretory substance, being conspicuously coloured, would also pigment the wings. The wings of *Vanessa io*, generally considered as not containing pteridines (Müller, 1956), show small yellow patches in their vivid mosaic of colours; patches which were found to contain xanthopterin. This limited, localized appearance of a pteridine as a wing pigment suggests that the deposition of pteridines in insect cuticular scales could have developed in connexion with a selective advantage in a pteridine-based pigmentation pattern, rather than in dry excretion. To what extent the latter has overtaken the former in the Pieridae, in which much larger amounts of pteridine are found in the cuticular scales, is a problem which needs further investigation.

If pteridines were synthesized to perform the role of pigmentation, it would be expected that their synthesis would take place late in metamorphosis as is the situation with other pigments. If, however, the pteridines were metabolic end-products, a gradual accumulation throughout metamorphosis would be expected. Furthermore, if the total amount of pteridine in the wing scales was found greatly to exceed the amount necessary for normal wing colour, a secondary excretory function for the pigments would be strongly suspected.

Whether 'intended' as pigment or as storage excretion, the pteridine content of the cuticular scales of the Pieridae is very high, representing a sizable percentage of the animals' complement of highly oxidized nitrogen (Harmsen, 1966*b*). It seems pertinent that the same questions should be asked for these pteridines as for the excreted ones. Are they synthesized in the general pool as nitrogen metabolism end-products adapted for a specialized excretory mechanism, or are they end products of functional pteridines that happen to be suitable for this specialized excretory mechanism?

Animal species can be divided into those producing or containing the physiologically active pteridines, and those with up to large quantities in excess of the necessary functional amounts. With this in mind, it seems plausible that, aside from one or more general functions (such as energy transfer, cell division, nucleic acid synthesis, etc.) for which small amounts of pteridines are necessary in all tissues (Jacobson, 1959), pteridines have taken on in some cases a function of pigmentation or excretion or both. This would account for those insects which produce quantities of pteridines greatly in excess of the physiologically functional ones, i.e. Becker's 'pteridine-containing' insects (Becker, 1937*b*).

MATERIAL AND METHODS

Experimental specimens

Pieris brassicae L. was mainly used in the experimental work as it is one of the classical examples of a 'pteridine-containing' insect. The specimens were taken from the same laboratory colony as the ones used for the recently published quantitative work on the pteridines in this insect (Harmsen, 1966*a*, *b*). In the remaining experiments, *Vanessa io* L. was used as a typical example of a 'pteridine-lacking' insect closely related to *Pieris*. The specimens were taken from a culture at the Virus Research Station of the Agricultural Research Council, Madingley Road, Cambridge. The caterpillars of this species were fed on the nettle *Urtica major* L. and the pupae were kept under conditions similar to those of *Pieris* (Harmsen, 1966*b*). The bugs, *Oncopeltus fasciatus* (Dall.), *Dysdercus fasciatus* Sign., and *Phonoctonus nigrofasciatus* Stål.; the locust *Schistocerca gregaria* (Forsk.); the flies, *Drosophila melanogaster* Mg., and *Calliphora erythrocephala* Meig.; and the beetle *Tenebrio molitor* L. were taken from cultures maintained at the Zoological Laboratory, Downing Street, Cambridge.

Extraction, separation and chromatography

The methods used for the extraction and separation of fluorescing and UV-absorbing substances have been described in detail in recent publications (Harmsen, 1966*a*, *b*).

Fluorimetric and spectrophotometric measurement

The method used for fluorimetric quantitative measurement is the one described by Kühn (1955). The detailed application of this method to the present problem has been described previously (Harmsen, 1966*b*). The spectrophotometric measurement of purines involved elution of the substance from the chromatogram and measurement at 265 mμ.

Total nitrogen assay

The technique used was basically that of Shaw & Beadle (1949). A few modifications in apparatus were necessarily incorporated before successful results were obtained. The meconium was dissolved in 0·3 ml. of a 0·02% lithium carbonate solution in 0·5 N ammonia, and 3·73 μl. samples of this solution were placed in the digestion tubes. The tubes were dried overnight in a 105° C. oven before the digestion mixture was added. This drying was necessary to remove the ammonia. Diffusion dishes were turned out of solid brass in place of using the suggested ones with separate sides and bottoms. A more accurate and more easily manipulated microburette than the one described by Shaw and Beadle was used for the titration. It was constructed from a mercury-filled screw micrometer attached to a thin glass capillary filled with the standard base. All measurements made with this technique were below 4 μg. total nitrogen, which is well below the maximum (approximately 10 μg.) allowed.

Quantitative expression and comparison

A detailed description of the methods and considerations regarding quantitative expression and comparison has been published recently (Harmsen, 1966*b*).

Dissection techniques

Immediately after pupation, the pupal cuticle of *Pieris brassicae* is still soft and pliable, yet very strong, much like a rubber or plastic sheet. In this early stage, it is possible to roll the wings one by one away from the body. A ligature can be applied around the base of the wing, which then can be removed without causing any bleeding. To prevent evaporation through the exposed thin cuticle normally underlying the wings, a layer of low melting point wax or a smear composed of pyroxylin and castor oil dissolved in a mixture of ethanol, ethyl acetate, butanol and amyl acetate (commercially available under the name 'new skin') can be applied.

Using this method, butterflies may be produced which develop normally in every respect except for the absence of one or two wings. When three or four wings are removed, the animal remains alive for up to 7 days, but dies before emergence.

RESULTS

Excretion and storage in Pieris brassicae

It has been shown (Harmsen, 1966*b*) that the larvae of *Pieris* do not excrete significant amounts of pteridine. Only during the last 24 hr. before pupation are small quantities of simple pteridines excreted. These quantities do not exceed 4% of the estimated intake of dietary folic acid and riboflavin. It seems that the entire simple pteridine content of the fully grown larva (approx. 135 μg.) could be accounted for by dietary intake and oxidation of the conjugated pteridines and riboflavin (Blair, 1961; Harmsen, 1965*b*; Nathan, Hutner & Levin, 1956). Most of these simple pteridines are stored in the integument and the fatbody of the pupa.

Of the large amounts of pteridine accumulated in the pupa (560 μg.) only traces are excreted with the meconium (approx. 2%), while the remainder is stored in the body: 25–30% in the living part of the body, 70–75% in the cuticular scales. Therefore, at no stage of the development of *Pieris* are the pteridines a true excretory product (Harmsen, 1966*b*).

An interesting shift in purine excretion occurs at about the time of pupation. Uric acid is the main end-product of nitrogen metabolism in the larva, and most of this substance is excreted steadily with the larval excreta at a rate of over 1 mg./24 hr. during the fifth instar. In the prepupa, however, uric acid excretion decreases rapidly, and throughout the pupal period the amount of uric acid remains constant at 1·5–2 mg. or increases only very slightly. The general end product of nitrogen metabolism during this stage is xanthine which accumulates quickly to a quantity of approximately 1·2 mg. On emergence of the adult, only 20% of the uric acid is excreted, the remainder being stored mainly in the fatbody. No xanthine is excreted; it is quantitatively stored in the fatbody (Harmsen, 1966*b*).

Other nitrogen-containing substances are encountered both in the larval excreta and in the meconium. Of these substances, kyurenine is the most important, occurring in quantities of some 100 μg. per 24 hr. during the fifth larval instar, and approximately 50 μg. in the meconium. Kynurenine is excreted quantitatively; no storage was found in the pupa.

Amputation of pupal wings

Larvae of *Pieris brassicae* were weighed at the spun-down prepupal stage and allowed to pupate. Immediately after pupation one forewing was rolled back, ligated and amputated. The exposed underwing was covered with a hydrophobic substance (see p. 6) to prevent loss of pupal water, and the pupae were stored from 7 to 8 days at 26° C. and 62 % R.H. On emergence, the meconium was collected quantitatively and the fully expanded adults (with only three wings) were separated into two parts: wings, and body. Each of these parts was extracted in the normal way and chromatographed. The pteridines were measured fluorimetrically. Six male insects of approximately similar weight were used. The results are summarized in Table 1.

Table 1. *Effect of wing amputation on pteridine synthesis and localization in* Pieris brassicae *L.* (*expressed in micrograms per average of six specimens*)

		Wings	Body	Meconium	Total
Experimental (three-winged) adult	Isoxanthopterin	33	17	0·6	51
	Leucopterin	169	88	9·6	256
Normal (four-winged) adult	Isoxanthopterin	44	17	0·4	62
	Leucopterin	239	69	6·5	314

The measurements of wing pteridine content coincide well with the expected 25 % drop as a result of the removal of one of the four wings. The blocking of the deposition of these pteridines in the wings has two very different effects. The pteridine content of both the body and the meconium shows a significant increase, but this only partly compensates for the loss of wing pteridines. The result is a considerable overall reduction in the pteridine content. Thus, there is not only a redistribution of the pteridines, but also an inhibition of synthesis. The very minor increase in pteridine content (when expressed in micrograms) of the meconium shows that the excretory system must possess a threshold for pteridines higher than the haemolymph concentration, even when there is an obvious trend towards 'overproduction'.

In the stock colony of *Pieris brassicae* the variety *cerulea* appeared as a spontaneous mutation. This mutant only became available after the experimental work on quantitative pteridines in *Pieris* was completed. Consequently, no accurate measurements are available for the pteridine content of this variety. The total absence of both xanthopterin and erythropterin (Harmsen, 1964), though interesting in itself, has little bearing on the present subject. Of more direct interest is the total reduction in pteridine content, due to the partial absence of wing scales coupled with a small but significant increase in pteridine content of the meconium. This situation in var. *cerulea* is more or less a natural confirmation of the experimental results obtained in the wing amputation experiments.

Pteridine storage and excretion in Vanessa io

A series of *Vanessa io* larvae of approximately the same age and size were reared and allowed to pupate. Just before pupation, a sample of prepupae was homogenized in the standard way and centrifuged (Harmsen, 1966*b*). Chromatograms were prepared from the supernatant of the homogenate. A second sample, this time of pharate adults just prior to emergence, was treated in the same way. The remainder of the

group was allowed to emerge, at which time the meconium was collected to be treated separately. These adults were killed after 24 hr. and separated into wings and body.

The chromatograms of all these samples showed a pteridine composition qualitatively similar to that of *Pieris brassicae*. Müller (1956) made a qualitative survey of the fluorescing substances of *Vanessa io* and reported finding isoxanthopterin, riboflavin and five unidentified substances. In the present investigation, leucopterin, isoxanthopterin, sepiapterin (in the prepupa), xanthopterin (in the wings of the adult) and kynurenine (in the meconium) were identified. Only leucopterin and isoxanthopterin are of quantitative significance in *Vanessa*. These latter two pteridines were measured quantitatively. No attempt was made to measure the other fluorescing or UV-absorbing substances, but a general estimate of the concentration was made. The results are contained in Table 2. Since the weight distribution curves of *Pieris* and *Vanessa* overlap considerably, the 'standard' *Vanessa* is equal in weight to the 'standard' *Pieris*. In this way comparison was made much easier.

Table 2. *Localization of pteridines and purines in* Vanessa io *L.* (*expressed in micrograms per 'standard' individual*)

	Leuco-pterin	Isoxantho-pterin	Other pteridines	Total pteridines	Uric acid	Other purines	Kynu-renine
Prepupa	31·3	11·5	−	45	++	−	−
Pharate adult	40·8	17·2	+	60	+++	+	+
Meconium	17·1	7·2	−	25	+++	o	+
Wing scales	—	—	—	—	—	o	o
Body	21·3	9·1	+	32	++	−	−

Rough expression: o no measurable amount, − traces, + small amounts, ++ large amounts, +++ very large amounts.

Both the pteridine content of the prepupa and the rate of synthesis in the pupa and pharate adult are much lower than in *Pieris*. As a result, the adult contains only about one-tenth as much pteridine as does *Pieris*; an actual amount of approximately 60 μg. which is comparable to the amount of kyurenine at the same stage.

The other major difference between *Vanessa* and *Pieris* is in the distribution of the pteridines. Practically no pteridines are deposited in the wings of *Vanessa*, and nearly half of the total pteridine content of the emerging adult is excreted with the meconium. On the other hand, 70–75 % is stored in the wings of *Pieris* and only 3 % is excreted. If one assumes that the total amount of highly oxidized, nitrogen-containing substances synthesized during the life history of *Pieris* and *Vanessa* are approximately the same (no direct measurement was made for *Vanessa*), one can then conclude that the pteridines in *Pieris* account for 10–15 % of the total oxidized nitrogen, while in *Vanessa* they account for only 1·0–1·5 %. Furthermore, *Vanessa* seems to synthesize uric acid throughout its entire life, while *Pieris* changes to xanthine synthesis at the time of pupation.

Pteridine content of other insects

In no group of insects, other than the Pieridae, has the description of pteridines included a quantitative estimate. From the extensive work on *Drosophila melanogaster* it can be deduced that this fly contains comparatively large amounts of pteridines;

however, no quantitative work as such has been attempted with this species. In an effort to obtain a rough estimate of total pteridine content, various insects were extracted and chromatographed on a quantitative basis. The following insects were examined:

Orthoptera	*Schistocerca gregaria* (Forsk.)
Hemiptera	*Dysdercus fasciatus* Sign.
	Oncopeltus fasciatus (Dall.)
	Phonoctonus nigrofasciatus Stål.
Coleoptera	*Tenebrio molitor* L.
Diptera	*Calliphora erythrocephala* Meig.
	Drosophila melanogaster Mg.

Schistocerca contains fairly large quantities of xanthopterin and sepiapterin in the mature male. The yellow pigmentation, however, is mainly due to a chloroform-soluble substance. In relation to body weight, the amount of pteridine is considerably less than in *Pieris*, but somewhat higher than in *Vanessa*. The pteridine content of *Schistocerca* is quantitatively speaking more or less comparable to that of the larva of *Pieris*.

The three bugs contain large quantities of erythropterin in the integument, and of xanthopterin in both the integument and fatbody. Bartel, Hudson & Craig (1958) failed to locate erythropterin in *Oncopeltus*, probably because of their harsh extraction methods. The total quantity of pteridine in these insects is very high (much the same order of magnitude as in adult *Pieris*).

The pteridine content of *Tenebrio* is extremely low. Only faint traces of isoxanthopterin and biopterin (?) could be located. It may be significant to note that *Tenebrio* is very slow growing: 9–12 months per generation.

Qualitatively, *Calliphora* and *Drosophila* differ little in pteridine content. The main difference is quantitative. Whereas *Drosophila* is characterized by a noticeably high content of the drosopterins, *Calliphora* contains relatively large amounts of sepiapterin and isosepiapterin, as in the mutant *sepia* of *Drosophila*. Both species contain large amounts of isoxanthopterin, and smaller amounts of other pteridines. In relation to body weight, the total pteridine content is much higher in *Drosophila* than in *Calliphora*. On the other hand, *Calliphora* meconium has a higher pteridine content than *Drosophila* meconium.

The total pteridine content of these insects could not be measured fluorimetrically, since no standard curves were available for the drosopterins, sepiapterins and erythropterin, which were unavailable in chemically pure form.

Purines and pteridines in total nitrogen excretion

The specimens of *Pieris brassicae* used in this section of the experimental work are of the same series as those used in the work on meconial excretion and storage in the adult (Harmsen, 1966*b*). The average total pteridine content of the meconium of this series was 12 μg. (representing 4 μg. of nitrogen); the average total purine content was 456 μg. (representing 153 μg. of nitrogen); furthermore, 50 μg. of kynurenine was found (which accounts for only 7 μg. of nitrogen). Thus all these substances account for a total nitrogen content of 164 μg. in the meconium. In the body and wings of the

adult, 549 μg. of pteridine accounts for 197 μg. of nitrogen, and 2960 μg. of uric acid and xanthine for another 987 μg.

These figures show that the meconial excretion represents only a small part of the total oxidized, non-functional nitrogen (2·0% of the pteridine nitrogen and 13·4% of the purine nitrogen). Nevertheless, the excretion includes practically all the kynurenine. Therefore, it seemed essential to discover whether any non-fluorescing, non-absorbing substances are present in significant amounts in the meconium and, if so, how much nitrogen they represent.

Samples of meconium were analysed with the ultra-micro Kjeldahl technique of Shaw & Beadle (1949). The results gave a nitrogen normality of 0·0545 to 0·0592 (av. 0·0577) for the samples. As the meconium was dried and subsequently dissolved in 0·3 ml. of distilled water, a normality of 0·0577 means that the average meconial excretion of this series contained

$$0{\cdot}3 \times 14 \times 0{\cdot}0577 \times 1000 = 241\ \mu g.\ \text{of nitrogen.}$$

The pteridines, purines and kynurenine were found to account for 164 μg. of excreted nitrogen; thus other unidentified substances must account for approximately a further 75 μg. of nitrogen in the meconium.

Table 3. *Distribution of total oxidized nitrogen in* Pieris brassicae *L.* (*expressed in micrograms and in percentage of total*)

	Purine		Pteridine		Urea		Kynurenine		Total	
	μg	%	μg	%	μg	%	μg	%	μg	%
Stored in adult	987	69	197	14	—	—	—	—	1184	83
Meconium	153	11	4	0·3	50*	3	7	0·5	241	17
Total	1140	80	201	14	50*	3	7	0·5	1425	100

— No measurable amount. * Approximation.

Chromatograms containing approximately one-tenth of an individual's meconium run in 70% *n*-propanol were sprayed with a standard dimethyl-amino-benzaldehyde reagent which showed the presence of urea in a concentration approximately similar to that found on control chromatograms containing 12 μg. of urea. This means that urea accounts for the greater part of the so far 'unaccounted for' nitrogen. Similar chromatograms of wings and body of *Pieris* showed no measurable amount of urea.

From these results it can be concluded that the minor metabolic end-products, i.e. urea and kynurenine, are quantitatively excreted; the major nitrogen-containing metabolic end-products, i.e. purines and to a lesser extent pteridines, are only partly excreted, the greater part being stored in the body. A summary of the distribution of the total oxidized, non-functional nitrogen is found in Table 3.

DISCUSSION

In most species of insects, uric acid or allantoin are the excretory end-products of general nitrogen metabolism. Substances such as kynurenine and urea are also produced in all investigated species, but in much smaller amounts. They are most probably end-products of the specialized pool. The pteridines of the majority of

insects also belong to this category. They are the oxidized, excretory end-products not only of the physiologically active pteridines, but possibly also of pyrimidines, as a relatively large amount of pyrimidines may well be released on the breakdown of larval tissues. However, it is obvious that in some groups of insects, notably in the Pieridae as exemplified by *Pieris brassicae*, the pteridine content is much higher than can be accounted for in this way. A further complication is the storage of substances normally accepted as excretory products. The storage of pteridines need be no obstacle in considering these substances excretory, since, for example, 80% of the pupal uric acid which most certainly is an excretory product, is stored in the adult of *Pieris*. Substances such as urea and kynurenine are never stored in adult insects because of their solubility in aqueous body fluids.

Pieris shows an increased pteridine production coupled with a specialized form of storage. A total amount of 560 μg. of pteridine accumulates during post-embryonic development, compared to the more usual figure of 60 μg. as found in *Vanessa*. Also, *Pieris* excretes a much smaller percentage (2%, i.e. 12 μg.) of its pteridines, while *Vanessa*, a more 'typical' insect, excretes approximately 40% (i.e. 25 μg.). Furthermore, the situation in *Pieris* is complicated in that the pteridines are irreversibly stored in the cuticular wing scales, which thereby acquire a definite pigmentation. Lastly, this species is unique among the investigated insects in switching from uric acid to xanthine synthesis at the time of pupation, the xanthine being stored quantitatively in the adult.

The situation in *Pieris* shows that the Pieridae have evolved simultaneously a specialized mechanism of formation of nitrogen-metabolism end-products, i.e. xanthine and leucopterin, and an improved storage potential for these substances. The wing-ligation experiments show that an effective barrier exists which prevents the excretion of pteridines. As it is improbable that *Pieris* would use ten times as much physiologically active pteridines as, for example, *Vanessa*, the tenfold increase in oxidized, simple pteridines must indicate that pteridine synthesis in *Pieris* is indeed extended to form a part of the general nitrogen excretory mechanism.

For those insects which store purines and/or pteridines in the fatbody, it could be maintained that this storage is potentially reversible. An adult with a long life span, during which it feeds exclusively on nectar and dew, may well benefit from a store of nitrogen-containing substances. There is some evidence that insects can utilize stored uric acid, and re-introduce the nitrogen into the general pool (Berridge, personal communication). The shift to xanthine production in *Pieris* may be an adaptation in this direction. However, this argument does not hold for the irreversibly stored pteridines and uric acid in the cuticular wing scales of the Pieridae.

In the introduction the problem was recognized of having to decide whether the increased pteridine content of *Pieris* was due to the use of pteridines as wing pigments, or to a more highly developed system of storage excretion. The experimental evidence strongly supports the storage-excretion hypothesis, although it is recognized that pteridines have a secondary function of pigmentation. Typical pigments such as melanins and ommochromes are synthesized shortly before they function as pigments. Pteridines, on the other hand, are synthesized continually throughout development and, in *Pieris*, in quantities greatly in excess of the amount necessary to provide full pigmentation. Furthermore, the pteridine content not only of the wings, but also of

the body of *Pieris* is much higher than in other insects. The *Vanessa* body contains approximately 32 μg. of total pteridine; the *Pieris* body as much as 150 μg. This considerable increase does not in any way affect the pigmentation of the body of *Pieris*.

Thus, in the Pieridae as represented by *P. brassicae*, the 'extra' pteridines are stored excretory products of the general nitrogen pool, with a localized secondary effect in pigmentation. Whether this pigmentation effect has any selective advantage is a difficult question in the case of *Pieris*, as in this species the pteridines are not arranged in any definite pattern. However, the deposition of pteridines in the wings of some other Pieridae (e.g. *Euchloe*) result in a very marked colour pattern suggesting the existence of a selective advantage in pteridine pigmentation. It is therefore valid to consider the pteridines as excretory products which function in ornament, rather than excretory products with an incidental ornamental effect.

SUMMARY

1. Previous work on the storage excretion theory of pteridines is summarized briefly, and the controversial aspects of this theory are discussed.

2. A general model for all nitrogen-containing substances in an animal body is presented. The possible pathways for pteridines in this model are discussed, and the storage excretion hypothesis is related to the model.

3. Storage and excretion of nitrogen-containing substances in *Pieris brassicae* were compared chromatographically on a quantitative basis. Only traces of the pteridines are excreted, the majority being stored, mostly in the adult cuticular scales. Only 20% of the uric acid is excreted; the xanthine is quantitatively stored in the fatbody.

4. Removal of part of the wing storage potential increases excretion and fatbody storage only very slightly. There is a reduction in overall pteridine synthesis.

5. A partly scaleless mutant, *cerula*, of *P. brassicae* also shows a slight increase in pteridine excretion coupled with an overall decrease in pteridine synthesis.

6. *Vanessa io*, a related species not containing large quantities of pteridines, was closely compared with *P. brassicae*. In *Vanessa* nearly half the pupal pteridines are excreted with the meconium, and no storage is found in the cuticular scales.

7. Several insects of other orders were investigated quantitatively. Only in some Hemiptera were pteridines found, stored in quantities comparable to those in Pieridae.

8. Using an ultra-micro Kjeldahl technique for total soluble nitrogen, the relative role of purines, pteridines, and some other minor substances, both in storage and excretion, was established.

9. It is obvious that in Pieridae the pteridines account for a much higher percentage of the total highly oxidized nitrogen produced (14%) than they account for in most other insects. The Pieridae differ further in storing most of these pteridines irreversibly in the cuticular scales of the adult.

10. The evidence strongly suggests that the Pieridae have evolved a metabolic mechanism of which pteridines are the general end-product as well as a mechanism of dry storage excretion for these end-products.

11. The pigmentation effect of cuticular pteridine deposition is considered a side-effect.

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REFERENCES

BARTEL, A. H., HUDSON, B. W. & CRAIG, R. (1958). Pteridines in the milkweed bug, *Oncopeltus fasciatus* (Dallas). I. Identification and localization. *J. Insect Physiol.* **2**, 348–54.

BECKER E. (1937*a*). Über das Pterinpigment bei Insekten und die Färbung und Zeichnung von *Vespa* im Besonderen. *Z. Morph. Ökol. Tiere*, **32**, 672–751.

BECKER, E. (1937*b*). Das Fehlen der Pterine in den Exkrementen pterinführender Insekten. *Hoppe-Seyler's Z. physiol. Chem.* **246**, 177–80.

BLAIR, J. A. (1961). Conversion of 2-^{14}C-pteroyl-L-monoglutamic acid into isoxanthopterin in *Drosophila melanogaster*. *Nature, Lond.*, **192**, 757–8.

HARMSEN, R. (1963). The storage and excretion of pteridines in *Pieris brassicae* L. and some other insects. Thesis, University of Cambridge.

HARMSEN, R. (1964). Genetically controlled variation in pteridine content of *Pieris brassicae* L. *Nature Lond.*, **204**, 1111.

HARMSEN, R. (1966*a*). Identification of fluorescing and u.v.-absorbing substances in *Pieris brassicae* L. *J. Insect Physiol.* **12**, 23–30.

HARMSEN, R. (1966*b*). A quantitative study of the pteridines in *Pieris brassicae* L. during post-embryonic development. *J. Insect Physiol.* **12**, 9–22.

HOPKINS, F. G. (1895). The pigments of Pieridae: a contribution to the study of excretory substances which function in ornament. *Phil. Trans.* B. **186**, 661–82.

JACOBSON, W. (1959). Die Rolle der Folinsäure bei der Zellteilung. *Mitt. naturf. Ges. Bern*, **17**, 55–67.

KOSCHARA, W. (1936). Isolierung eines gelben Farbstoffs (Uropterin) aus Menschenharn. *Hoppe-Seyler's Z. physiol. Chem.* **240** 127–51.

KOSCHARA, W. & HAUG, H. (1939). Über die physiologische Bedeutung des Uropterins. *Hoppe-Seyler's Z. physiol. Chem.* **259**, 97–112.

KÜHN, A. (1955). Fluorometer for paperchromatograms. *Naturwissenschaften*, **42**, 529–30.

KÜRSTEINER, R. (1961). Ueber die fluoreszierenden Stoffe (Pterine) in den Meconien der Wildrasse und der Mutanten *White* und *Rosy*^{1} von *Drosophila melanogaster*. *J. Insect Physiol.* **7**, 5–31.

MÜLLER, G. (1956). Fluoreszierende Stoffe in *Vanessa io* L. chromatographisch untersucht. *Z. Naturf.* **11***b*, 221–2.

NATHAN, H. A., HUTNER, S. H. & LEVIN, H. L. (1956). Independent requirements for 'Crithidia Factor' and folic acid in a Trypanosomid flagellate. *Nature, Lond.*, **178**, 741–2.

RAUEN, H. M. & HALLER, C. V. (1950). Über die Ausscheidung von Xanthopterin nach Verabreichung von Pteroylglutaminsäure beim Menschen. *Hoppe-Seyler's Z. physiol. Chem.* **286**, 96.

SCHÖPF, C. & WIELAND, H. (1926). Über das Leukopterin, das weisse Flügelpigment der Kohlweisslinge (*Pieris brassicae* und *P. napi*). *Ber. dtsch. chem. Ges.* **59**, 2067–72.

SHAW, J. & BEADLE, L. C. (1949). A simplified ultra-micro Kjeldahl method for the estimation of protein and total nitrogen in fluid samples of less than 1·0 μl. *J. Exp. Biol.* **26**, 15–23.

WIELAND, H., METZGER, H., SCHÖPF, C. & BÜLOW, M. (1933). Über Leukopterin, das Flügelpigment der Kohlweisslinge (Pieriden). II. *Justus Leibigs Annln Chem.* **507**, 226–65.

WIGGLESWORTH, V. B. (1925). Uric acid in Pieridae: a quantitative study. *Proc. R. Soc.* **97**, 149–55.