SOME BIOCHEMICAL ASPECTS OF TYROSINE AMINOTRANSFERASE (L-TYROSINE: 2-OXOGLUTARATE AMINOTRANSFERASE, E.C. 2.6.1.5) (TAT) IN THE STAGES OF DEVELOPMENT OF *AEDES AEGYPTI* LINNAEUS.

by

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ABSTRAK

Variasi tirosine-2-oksoglutarat-aminotransferase (EC.2.6.1.5), TAT, dan protein terlarutkan telah disusuli sepanjang perkembangan pos-embrionik dan tumbesar dewasa muda untuk nyamuk, Aedes aegypti (Linnaeus) itu. Satu kesepadanan yang rapat antara aktiviti enzim dan kandungan protein terlarutkan telah diperhatikan semasa peringkat-peringkat takmatang untuk perkembangan. Aktiviti kemuncak untuk transaminase telah ditemui dalam anak-jelmaan larva pertengahan yang keempat dan ini diikuti oleh satu kemerosotan dalam aktiviti sebelum pupasi. Pada aktiviti maksimumnya, enzim serangga itu berbanding baik dengan enzim hati tikus dewasa dari segi aktivitinya. Enzim larva kononnya memamerkan satu ritma aktiviti harian, yang dicirikan oleh satu pertambahan mantap dalam aktiviti dari 06^{00} jam dan sampai aktiviti maksimum pada 24^{00} jam.

Variasi aktiviti TAT dalam ekstrak-ekstrak taktulin dari larva keseluruhan dengan jarak-panjang masa pengeraman, volum ekstrak, suhu, pH, kepekatan substrat dan piridoksal fosfat, telah dikaji. K_m untuk tirosine telah dijumpai sebagai 1.4×10^{-3} M dan untuk a-ketoglutarat, 7.45×10^{-4} M.

Dalam eksperimen-eksperimen pengemparan diferensial yang dilaksanakan atas enzim larva, kira-kira 50-60% aktiviti telah didapati semula dalam pecahan terlarutkan sementara pecahan mitokondria mempunyai aktiviti yang boleh diabaikan.

Elektroforesis gel poliakrilamid untuk TAT nyamuk membongkarkan kewujudan isozim-isozim. Perbezaan-perbezaan bersandar-usia dalam pola isozim telah dikesan. Jadi, enzim dewasa, enzim pupa, dan enzim larva didirikan oleh tiga, dua dan satu jalur, masing-masing. Perbezaan-perbezaan kuantitatif dalam pola-pola isozim telah juga diperhatikan.

Analog hormon juvenil, ZR-515 (isopropil 11-methoksi-3,7,11-trimetil dodeka-2,4-dieonat), dan siklik 3,5'-adenosine monofosfat tidak mempengaruhi aktiviti enzim anak-jelmaan larva yang keempat itu secara signifikan samada dipakai in vivo atau in vitro.

Dekapitasi mengakibatkan dalam pertambahan tiga-dan-empat kali dalam aktiviti TAT untuk serangga dewasa jantan dan betina yang bermuncul secara baru, masing-masing, tetapi gagal meningkatkan aktiviti serangga berusia tiga-dan-tujuh hari untuk samada jantina. Satu homogenat kepala yang disediakan dari dewasa-dewasa betina yang bermuncul secara baru menurunkan aktiviti enzim larva sebanyak 65.0% dan aktiviti enzim hati tikus dewasa sebanyak 19.3%. Komponen penghalangan didapati dalam supernatan selepas pengemparan 10,000 g selama 15 minit dan adalah haba-stabil dan terdialisiskan.

ABSTRACT

The variation of tyrosine-2-oxoglutarate amino-transferase (EC.2.6.1.5), TAT, and soluble protein was charted throughout the post-embryonic development and young adult growth of the mosquito, Aedes aegypti (Linnaeus). A close correspondence between the enzyme activity and soluble protein content was observed during the immature stages of development. Peak transaminase activity was encountered in the middle fourth larval instar followed by a decline in activity prior to pupation. At its maximum activity, the insect enzyme compared well with the adult rat liver enzyme in activity. The larval enzyme appeared to exhibit a diurnal rhythm of activity characterized by a steady increase in activity from 06^{00} hours to reach maximum activity at 24^{00} hours.

The variation of TAT activity in crude extracts from whole larvae with length of incubation time, volume of extract, temperature, pH, substrate concentration and pyridoxal phosphate PALP was studied. The Km for tyrosine was found to be 1.41×10^{-3} and for α-ketoglutarate, 7.45×10^{-4} M.

In differential centrifugation experiments conducted on the larval enzyme, roughly 50-60% of activity was recovered in the soluble fraction while the mitochondrial fraction had negligible activity.

Polyacrylamide gel electrophoresis of mosquito TAT revealed the existence of isozymes. Age-dependent differences in the isozyme pattern were detected. Thus, the adult, pupal and larval enzymes comprised

three, two and one bands, respectively. Quantitative differences in the isozyme patterns were also observed.

The juvenile hormone analogue, ZR-515 (isopropyl 11-methoxy-3,7,11-trimethyl dodeca-2,4-dieonate), and cyclic 3,5'-adenosine monophosphate did not affect the activity of the fourth larval instar enzyme significantly whether applied *in vivo* or *in vitro*.

Decapitation resulted in three- and four-fold increases in the TAT activity of the newly-emerged male and female adult insects, respectively, but failed to elevate the activity of three- and seven-day old insects of either sex. A head homogenate prepared from newly-emerged female adults reduced the larval enzyme activity by 65.0% and the adult rat liver enzyme activity by 19.3%. The inhibitory component resided in the supernatant after centrifugation at 10,000 g for 15 minutes, was heat-stable and dialyzable.

LIST OF ABBREVIATIONS

BT	Blue Tetrazolium
BPB	Bromophenol Blue
c-AMP	Cyclic 3',5'-Adenosine Monophosphate
DIECA	Diethyldithiocarbamate
DOPA	Dihydroxyphenylalanine
NAD^+	Nicotinamide Adenine Dinucleotide
NBT	Nitroblue Tetrazolium
pHPP	para-Hydroxyphenylpyruvate
PMS	Phenazine Methosulphate
PALP	Pyridoxal 5'-phosphate
TEMED	Tetramethylethylenediamine
TAT	L-Tyrosine: 2-oxoglutarate aminotransferase, E.C.2.6.1.5
ZR-515	Isopropyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dieonate

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INTRODUCTION

The ability of insects to metabolize amino acids by transamination is no longer in doubt. Naturally, most of the detailed investigations have been directed at the most active transaminases, glutamate-oxaloacetate and glutamate-pyruvate transaminases. Tyrosine-2-oxoglutarate transaminase (TAT), with its relatively low activity, has been little studied despite its participation in the metabolism of an amino acid peculiarly important to the protective make-up of an insect, tyrosine. The enzymes of tyrosine metabolism have long been held synonymous with the enzymes of the constructive pathway leading to the formation of substrates for tanning and hardening of the exoskeleton, and, with the exception of a few notable examples, less attention has been accorded to the degradative pathways. It was the intention of the present investigation to look into some aspects of the crude insect transaminase, partly to see how it compared with the mammalian enzyme, and also to follow its activity through growth and metamorphosis with the view of studying its response to some physiologically-active substances during a critical stage of morphogenesis.

The mosquito, Aedes aegypti (Linnaeus) was selected for study owing to the ease with which it lent itself to culture. Moreover, it is an insect of utmost medical importance and information gained from this investigation would contribute towards a greater understanding of the metabolic events accompanying its growth and development.

A summary of the various aspects of TAT of Ae. aegypti (L.) investigated in this study is as follows:

1. a) Course of TAT activity during post-embryonic development and early adult growth. Selection of the stage of development with maximum activity as the source of the enzyme for later studies.

   b) Differentiation of the fourth (final) larval instar into seven sub-stages based on imaginal eye development, and determination of TAT activity of each sub-stage for a detailed picture of the enzyme variation during fourth instar development.

2. Characterization of some parameters affecting TAT activity, including diurnal variations in activity.

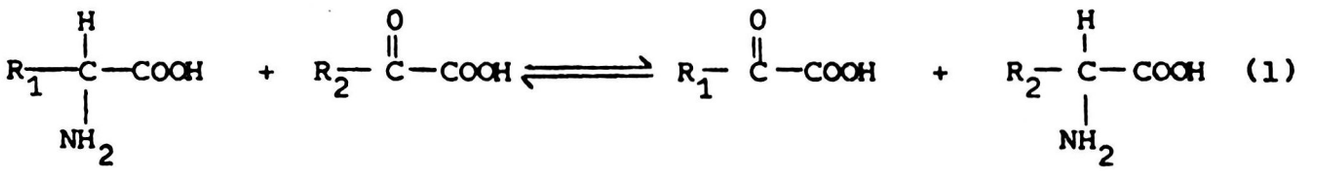
3. Differential centrifugation studies of TAT to determine its sub-cellular distribution.

4. Use of polyacrylamide gel electrophoresis for the detection of isozymic components and any qualitative changes in the isozymic pattern of the three main stages of growth, namely, larval, pupal and adult stages.

5. Response of TAT activity to the addition of a juvenile hormone analogue, ZR-515 (isopropyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate), cyclic 3',5'-adenosine monophosphate and an endogenous head factor obtained from the adult insect.

LITERATURE REVIEW

One of the key reactions in the metabolism of amino acids is the transamination reaction which comprises the transfer of amino groups from amino acids to keto acids without the intermediate formation of ammonia:



(1)

This transfer was reported by Chefurka (1965) to have been first effected in a model system containing boiling solutions of amino acid and keto acid by Herbst and Engel in 1934. In 1937, Braunstein and Kritzman demonstrated that the reaction also proceeded in many living organisms where it was catalyzed by enzymes. These enzymes have been variously termed transaminases, aminopherases and aminotransferases.

Since the discovery of Braunstein and Kritzman (1937), transaminases, as the enzymes will be called, capable of reacting with nearly all of the amino acids have been reported. Almost invariably, these reactions involved glutamic acid and ∝-ketoglutaric acid as one of the two substrate pairs:

L-glutamic acid + ∝-keto acid $\rightleftharpoons$ ∝-ketoglutaric + amino acid (2)

The central role of glutamic acid in the transamination reaction provides the prime mechanism for the synthesis of most of the non-essential amino acids in the cell capable of synthesizing the corresponding keto acids.

In addition, transaminases have been recognised to play an important role in glucogenesis and ketogenesis as well as in the degradative metabolism of waste nitrogen products.

The transamination process in insect tissues was first demonstrated by Barron and Tahmisan (1948). They established that the homogenates of leg muscles of Periplaneta could form alanine by the following reaction:

L-glutamic acid + pyruvic acid $\rightleftharpoons$ α-ketoglutaric acid + L-alanine (3)

In later experiments, it was established that the enzyme catalyzing this reaction, glutamate-pyruvate transaminase, is one of the two transaminases most active in the insect. The other transaminase is glutamate-oxaloacetate transaminase which catalyzes the reaction:

L-glutamate acid + oxaloacetic acid $\rightleftharpoons$ α-ketoglutaric acid + L-aspartic acid (4)

These two enzymes are, in fact, also the most abundant of the mammalian transaminases.

Thus, the glutamate-oxaloacetate transamination reaction, owing to its high activity, has been readily studied in a number of insects such as Schistocerca (Kilby and Neville, 1957), Musca (McAllan and Chefurka, 1961a; Price, 1961), Periplaneta (Wang and Dixon, 1960); McAllan and Chefurka, 1961b), Bombyx (Bheemeswar and Sreenivasaya, 1952; Shyamala and Bhat, 1955), Celerio (Belzecka et al., 1959; Raczynska-Bojanowska and Belzecka, 1962), Hyalophora (McAllan and Chefurka, 1961a), Calliphora (Desai and Kilby, 1958), Aedes (Murthy and Micks, 1964),

Tenebrio (Emmerich et al., 1965) and Galleria (Laviolette and Bonnot, 1966). Quantitative studies comparing the activity of this reaction with the glutamate-pyruvate reaction in Schistocerca (Kilby and Neville, 1957), Bombyx (Koide, F. et al., cited in Chefurka, 1965; Fukuda, 1957), Celerio (Belzecka et al., 1962) and Aedes (Murthy and Micks, 1964), have established that the glutamate-oxaloacetate reaction was often twice as active, but in Drosophila (Chen and Bachmann-Diem, 1964), Anthrenus (Nair and George, 1964), Pyrrhocoris (Nohel and Slama, 1972) and Locusta (Mordue and Goldsworthy, 1973), the reverse situation in which the glutamate-pyruvate reaction was the more active was true.

Contrary to the findings of Kilby and Neville (1957), Mordue and Goldsworthy (1973) found Schistocerca to possess larger quantities of the transaminase catalyzing the glutamate-pyruvate reaction, a difference which they attributed to dissimilarities in the kind of diet fed to the locusts (a "natural" as opposed to an "artificial" diet). This phenomenon has been encountered in vertebrates where changes in diet have been known to influence transaminase levels (Knox, 1964; Freedland et al., 1968). Also, it has been established that some aspects of intermediary metabolism such as lipid mobilisation, were different in locusts reared on fresh and artificial diets (Meyer and Candy, 1969; Goldsworthy et al., 1972).

Transamination rates with other amino acids as amino donors, and oxaloacetate, pyruvate or α-ketoglutarate as acceptors are about one-third to one-twentieth that of the glutamate-oxaloacetate reaction (Kilby and Neville, 1957; Desai and Kilby, 1958; Chen and Bachmann-Diem, 1964). In the shore crab, Carcinus maenus, only the glutamate-oxaloacetate and glutamate-pyruvate transaminase were sufficiently active for quantitative estimation which showed them to be of comparable activity (Chaplin et al., 1967). The glutamate-pyruvate transamination reaction has been reported in other crustaceans,

Eriocheir, Homarus and Astacus (Zandee et al., 1958) while the reaction between tyrosine and α-ketoglutaric acid has been encountered only in Carcinus.

The first demonstration of transamination between tyrosine and α-ketoglutarate in insects by the enzyme tyrosine-2-oxoglutarate transaminase (TAT) was made by Koide, F. et al. (cited in Chefurka, 1965) in Bombyx, followed by Kilby and Neville (1957) in Schistocerca, Desai and Kilby (1958) in Calliphora, Belzecka et al. (1962) in Celerio and Karlson and Sekeris (1962a) in Calliphora. In the survey of transamination reactions in Aedes aegypti (Murthy and Micks, 1964), no attempt was made to detect TAT. In many of the cases cited, TAT activity was shown to be low in comparison to that of glutamate-oxaloacetate and glutamate-pyruvate transaminases. Other than α-ketoglutarate, tyrosine can also transaminate to a limited extent with pyruvate but fails to do so with oxaloacetate (Chefurka, 1965). The ability of TAT to catalyze the reverse reaction, that is the synthesis of tyrosine from p-hydroxyphenylpyruvate (pHPP) and glutamate has been established in mammalian systems but similar information is lacking in insects.

In an examination of the reverse reaction:

$$\text{L-glutamate} + \text{pHPP} \rightleftharpoons \text{tyrosine} + \alpha\text{-ketoglutarate} \quad (6)$$

in the dog liver (Canellakis and Cohen, 1956), it was observed that the velocity of the reaction, although low, was a positive function of the glutamate concentration while pHPP displayed substrate inhibition at levels as low as 10^{-4} M. The same situation was encountered in partially purified rat liver (Kenney, 1959), in which the same low activities were found for the reverse reaction. It thus appears that in mammals, at least, contribution by the reverse reaction is negligible. An interesting point to note about the situation in insects is that neither glutamate

nor aspartate can transaminate with phenylpyruvic acid in tissues of Apis, Calliphora and Schistocerca (Zandee et al., 1958; Kilby and Neville, 1957).

In addition to the three most frequently encountered keto-acids, α-ketoglutarate, pyruvate and oxaloacetate, transaminations also occur with glyoxylic acid, to produce glycine (Chefurka, 1965) in the silk gland of Bombyx. The presence or absence of activity was related to the proportion of glycine found in the silk gland proteins, fibroin and sericin. Further, alanine was shown to be the most active amino group donor, a finding corroborated by Chen and Bachmann-Diem (1964) in Calliphora, while tyrosine was quite inactive. The glyoxylic acid-alanine reaction has also been observed in mammalian and avian tissues but the reaction in the silkworm was more easily detected and occurred at a higher rate.

Altogether, the effectiveness of some nineteen amino acids as amino donors has been established in insect transaminations. This number includes amino acids such as asparagine (Chefurka, 1965; Kilby and Neville, 1957) and ornithine (Kilby and Neville, 1957). This, together with other aspects of the intermediary metabolism of nitrogen compounds in insects has been reviewed in several articles (Chefurka, 1965; Chen, 1966; Gilmour, 1961; Kilby, 1963; Corrigan, 1970; Gilbert and Schneiderman, 1961; Gilbert, 1967).

In many of its properties, insect transaminases resemble the mammalian enzyme. For example, the pH optima of insect transaminases studied so far compare well with that of the mammalian enzyme (Meister,

1957). Generally, the insect glutamate-pyruvate transaminase has a rather broad pH range of 6.5 to 8.0, with an optimum at about 7.5 (Kilby and Neville, 1957; Desai and Kilby, 1958; McAllan and Chefurka, 1961b; Chen and Bachmann-Diem, 1964) and the glutamate-oxaloacetate and glyoxylate-alanine transaminases, pH ranges of 7.4 to 8.4 and 9.0 to 9.5 respectively (Chefurka, 1965). Bacterial transaminases attain peak activity at around pH 8.0 (Gunsalus and Stamer, 1955) while the rate of purified mammalian TAT reaches an optimum at pH 7.5 (Hayashi et al., 1967), pH 7.6 (Kenney, 1959) or pH 7.7 (Canellakis and Cohen, 1956). In most experiments involving the insect or mammalian enzyme, a working pH of 7.4 is used.

Transaminases, irrespective of origin, require pyridoxal phosphate as coenzyme to function effectively. Dialysis serves to remove the coenzyme resulting in a partial or total loss of enzyme activity. This has been demonstrated in practically all the vertebrate and bacterial transaminases so far resolved as well as in many insect species: Aedes (Murthy and Micks, 1964), Drosophila (Chen and Bachmann-Diem, 1964), Celerio (Belzecka and Raczynska-Bojanowska, cited in Chefurka, 1965), Periplaneta (McAllan and Chefurka, 1961b) and Calliphora (Sekeris and Karlson, 1962). In most of the cases cited, activity could be restored to varying degrees by supplementing pyridoxal phosphate to the dialyzed preparation.

Further evidence of the participation of pyridoxal phosphate in transaminase activity is the effect of carbonyl reagents, e.g., hydroxylamine, hydrazine and isonicotinylhydrazide on activity. These

reagents are known to inhibit B_6-catalyzed reactions, by reaction with pyridoxal phosphate (Lichstein, 1945; Schlenk and Snell, 1945) and, in transaminations, by gradual reaction with the keto-acid substrates (Gale, 1946; Blaschko, 1945). This inhibition has been observed in the major insect transaminases (Bheemeswar, 1959; Belzecka and Raczynska-Bojanowska, cited in Chefurka, 1965). TAT is also strongly inhibited by reagents which react with sulphydryl groups such as p-chloromercuriphenyl sulphonate, o-iodosobenzoate and iodoacetate. This inhibition can be almost completely prevented by cysteine or glutathione at 10^{-3} M (Kenney, 1959). Inhibition of TAT in crude rat liver preparations by L-thyroxine has been studied by Litwack (1957) but this inhibition can be reversed by increasing the amount of enzyme assayed. In addition, the hydroxylated derivatives of tyrosine (e.g. dopamine, norepinephrine, dihydroxyphenylacetic acid) and other aromatic compounds (e.g. p-hydroxyphenylacetic, phenylethylamine) (Jacoby and La Du, 1964) and p-fluorotyrosine (Canellakis and Cohen, 1956) compete effectively with tyrosine for the active site on the enzyme protein. There is also some evidence of heavy metal inactivation (Kenney, 1959).

Organ distribution studies of insect TAT are far behind those of other animals, especially the mammals. On the other hand, the organ distribution of the two most active transaminases in insects, glutamate-oxaloacetate and glutamate-pyruvate transaminases, has been worked out and has been found to be essentially the same: the Malpighian tubules, fat body, gut epithelium and somatic muscles are the main sites of activity. However, no one organ can be regarded as being the most active as this appears to vary in different insects. The more consistent of the two

enzymes, glutamate-pyruvate transaminase, occurs mostly in the Malpighian tubules and fat body and less in the gut. This has been established in Schistocerca (Kilby and Neville, 1957), Bombyx (Fukuda, 1957), Calliphora (Desai and Kilby, 1958) and Drosophila (Chen and Bachmann-Diem, 1964). The distribution of glutamate-oxaloacetate transaminase is more variable. On a wet weight basis, the Malpighian tubules and fat body are again the most active organs in Schistocerca (Kilby and Neville, 1957) and Periplaneta (McAllan and Chefurka, 1961a). The nerve cord, thoracic muscles and gut were also implicated as good sources of activity in Periplaneta while in the hawk moth, Celerio (Belzecka et al., 1959) and in Locusta (Mordue and Goldsworthy, 1973), activity in the muscle was, respectively, ten and three times higher than that in the fat body. In both the insects as well as Bombyx (Bheemeswar and Sreenivasaya, 1952) and Musca (Price, 1961), the haemolymph is devoid of activity. A high glutamate-oxaloacetate transaminase activity of the nervous system and muscle is also found in mammals (Cohen and Heklius, 1941) and crustaceans (Huggins, 1968).

Although transaminase activity has been demonstrated in the various insect tissues, the fat body has the widest range of transaminations of which those reactions involving glutamate have been found to have activities of the same order of magnitude as those in the rat liver (Awapara and Seale, 1952). An active site for the intermediary metabolism of amino acids, the fat body also fulfils many of the metabolic functions associated with the vertebrate liver (Kilby, 1963). The mammalian liver contains many of the transaminases catalyzing the reaction between glutamic acid and the keto acids corresponding to the

naturally-occurring amino acids (except possibly glycine, threonine and lysine). In addition to TAT, the other enzymes involved in the metabolism of tyrosine, such as p-hydroxyphenylpyruvate oxidase and homogentisate oxidase, are located primarily in the liver of the rat (Lin and Knox, 1958). Smaller amounts of TAT have also been detected in the heart and kidney (Lin and Knox, 1958) and adrenal and brain of the rat (Wurtman and Larin, cited in Iwasaki and Pitot, 1971), listed in order of activity. The same pattern of distribution has been found in the dog by Canellakis and Cohen (1956). They further compared the TAT activity of the liver from five species of vertebrates and showed that dog liver had maximal activity, followed by the rabbit. Pigeon liver is a relatively poor source. Owing to its low activity and in some cases, the limitation imposed by size, the tissue distribution of TAT in insects has not been studied and to date, all work has been confined to fat body or whole insect preparations.

The transaminases of plant and animal cells are found both in cytoplasm and mitochondria and the enzymes of each fraction are often characterized by properties peculiar to it. An example of this is hepatic TAT. A large proportion of TAT activity of liver cells is associated with the 'soluble' fraction of differential centrifugation studies, and to some extent, also with the mitochondria (Hird and Rowsell, 1950; Rowsell et al., 1963; Litwack et al., 1963; Fellman et al., 1969). Unlike 'soluble' TAT of rat liver, which can be induced by glucocorticoids and other agents, and has rigid substrate requirements, the mitochondrial enzyme is not subject to large changes in activity, has a broader tissue distribution and transaminates more readily with aspartate and alanine

than with tyrosine (Fellman et al., 1969; Miller and Litwack, 1971). In other studies where only two fractions are differentiated, namely, the residue and supernatant of 'high-speed' centrifugation (here taken to mean speeds necessary to separate mitochondria and larger particles from the microsomal and soluble fractions), TAT activity is found to be greater in the supernatant (Canellakis and Cohen, 1956). In this way, Sekeris and Karlson,(1962) showed the TAT of Calliphora larvae to be a soluble enzyme. The significance of this distribution lies in the fact that most of the cytoplasmic transaminases serve to gather amino groups of amino acids as glutamic acid which then enters the mitochondria to further transaminate with mitochondrial glutamate-oxaloacetate or glutamate-pyruvate transaminase. Alternatively, the glutamic acid can be oxidatively deaminated by mitochondrial glutamic dehydrogenase (Cohn and Stumpf, 1972). Further proof of this is provided by the finding that a mitochondrial transaminase exists which can function interchangeably with aspartate and alanine transaminases, thereby helping to regulate the major keto-acids, oxaloacetate, pyruvate and α-ketoglutarate, for oxidative metabolism, the enzymes of which also reside in the mitochondria (Miller and Litwack, 1971).

Besides tissue and sub-cellular location, hepatic TAT activity varies as a function of the time of day (Wurtman, 1968; Wurtman and Axelrod, 1967; Civen et al., 1967; Shambaugh et al., 1967). Beyond the 24-hour cycles, seasonal variations in activity have also been recorded for TAT (Chuah et al., 1971).

Another source of variation of enzyme activity is growth. In mammals, the livers of foetal rat and pre-mature human infant exhibit a

markedly reduced ability to metabolize tyrosine, in contrast to the much higher activity of livers obtained from neonatal rats and full-term infants. A well-studied example of this phenomenon, TAT, attains peak activity almost two-fold adult values twelve hours after birth (Sereni et al., 1959; Msuya and Schepartz, 1969). The reduced activity of the foetal liver has been ascribed to the lack of TAT (Kretchmer and McNamara, 1956).

A different situation is encountered in insects where the rigidity of the exoskeleton necessitates that growth be punctuated by a series of moults during which the exoskeleton is shed. In ametabolous and hemimetabolous insects, the adult stage is achieved gradually while in holometabolous insects, the pupal-adult transformation or metamorphosis is preceded by extensive tissue histolysis and histogenesis associated with imaginal differentiation. Energy metabolism, as measured by oxygen consumption, thus follows a characteristic U-shaped curve during pupal development, the fall in oxygen consumption corresponding with histolysis and the subsequent rise with histogenesis and differentiation, a pattern of activity also parallelled by the respiratory enzymes (Wigglesworth, 1954; Agrell, 1964). Qualitative and quantitative changes in lipid (Gilbert, 1967), carbohydrate (Gilbert, 1967); Karlson and Sekeris, 1964) and amino acid and protein content (Chen, 1966) related to metamorphosis have been demonstrated. In view of the profound changes occurring during metamorphosis, it has been confirmed that there is a disproportionately small change in the overall pool size of the free amino acids. Thus, only slight fluctuations corresponding to tissue dissolution and its subsequent reconstruction have been detected in the haemolymph of the pupa of

Calliphora (Agrell, 1949), Ephestia (Chen and Kühn, 1956), Culex (Chen, 1958), Prodenia (Levenbook, 1962), Phormia (Levenbook and Dinamarca, 1966) and Lucilia (Birt and Christian, 1969) while no major variations are apparent in Tenebrio (Patterson, 1957). Similarly, in the larva, development is accompanied either by a constant total concentration of haemolymph amino acids, or a steady decrease in the total concentration as development proceeds. The two exceptions are tyrosine and proline which always show a continuous increase. All the insect species investigated fall into one of the two categories: constant concentration - Culex quinquefasciatus, Aedes aegypti, Ephestia kuhniella, Culex pipiens, Schistocerca gregaria, Rhodnius prolixus; steady decline in concentration - Drosophila melanogaster, Bombyx mori, Corcyra cephalonica (Chen, 1966). The significance of tyrosine accumulation lies in the finding that it enters into important metabolic activities at the larval-pupal moult or metamorphosis, namely, its incorporation into the cuticular proteins shortly before pupation (Hackman, 1953a) and its role as the substrate for the tanning reaction accompanying puparium formation (Fraenkel and Rudall, 1940, 1947; Dennell, 1946, 1947, 1949; Pryor et al., 1947; Hackman, 1953b; Brunet, 1963; Cottrell, 1964; Gilmour, 1961). In the early larval stages, tyrosine is mainly transaminated with α-ketoglutarate and, subsequently, further degraded, while in older larvae, conversion to tanning substrates predominates (Karlson and Sekeris, 1962a, b; Sekeris and Karlson, 1962). Tyrosine metabolism in the Hemimetabola also depends on the stage of development. Between moults, phenolic acids are the main metabolites while the production of tanning substances prevails at and just after the moults (Karlson and Herrlich, 1965; Mills et al., 1967; Murdock et al., 1970; Hopkins et al., 1971). However, the enzyme system

responsible for the production of the phenolic acids is present throughout the life of the insect as determined by *in vitro* experiments (Mills and Lake, 1971). In the adults of both groups of insects, tyrosine is mostly degraded to phenolic acids with continued low level synthetic reactions occurring for wound healing and hardening of replacement cuticle and for the production of catecholamines (Hopkins *et al.*, 1971).

The role played by transaminases in the post-embryonic development of insects is thought to be one of maintaining a balanced amino acid pool for protein synthesis (Meister, 1957; Gilbert, 1967). This has been borne out by the findings of Belzecka *et al.* (1959) in *Celerio*, and McAllan and Chefurka (1961a) in *Musca* and *Hyalophora*. The latter workers found that glutamate-oxaloacetate transaminase activity during the growth of *Musca* larvae and pupae described the same U-shaped curve as did respiration and the respiratory enzymes. Further, Zwicky and Wigglesworth (1956) showed that the U-shaped oxygen consumption curve of *Rhodnius* reflected closely the changes effected by protein synthesis. Protein synthesis may also be linked with the high glutamate-pyruvate transaminase activity in the silk gland of *Bombyx* larvae. The concomitant increases in transamination rates and protein synthesis resulting in enhanced growth and silk production in the worms administered with chloromycetin are thought to be related events (Chefurka, 1965). It is therefore the contention of many workers that a direct relationship exists between transaminase activity and the rate of protein synthesis in many insects (Belzecka *et al.*, 1959; Chen, 1966; Gilbert, 1967; McAllan and Chefurka, 1961a; Mordue and Goldsworthy, 1973; Wang and Dixon, 1960). On the other hand, the transaminase activity of certain mammalian tissues demonstrates

an inverse relationship, to vary proportionately with the rate of catabolic processes instead (Nichol and Rosen, 1963; Ashmore et al., 1964; Wergedal et al., 1964; Knox and Greengard, 1965). A similar inverse relationship has been confirmed for glutamate-pyruvate transaminase activity in Pyrrhocoris (Nohel and Slama, 1972).

The allatectomy of the nymphs and adults of Periplaneta reduced glutamate-oxaloacetate transaminase activity in the thoracic muscle (Wang and Dixon, 1960). Further, the reduction was greatest in the nymphs and adult females where the corpora allata was required for morphogenesis and ovarian growth, respectively. This suggested that the corpora allata affected protein metabolism and, possibly, even transamination. Its effect on the various aspects of protein metabolism such as the production of intestinal proteases and vitellogenesis has been described by many workers including Thomsen and Møller (1963), Bodenstein (1953), Dadd (1961) and Vandenberg (1964a,b). Nevertheless, its effect on transamination is still far from resolved. For example, an increase in glutamate-oxaloacetate transaminase activity has been reported for Tenebrio pupae (Emmerich et al., 1965) while no change in the glutamate-pyruvate transaminase activity of last-instar larvae and female adults of Pyrrhocoris could be discerned when juvenile hormone analogues were applied (Nohel and Slama, 1972).

Although a clear-cut hormonal influence on transaminase activity has not been demonstrated in insects, TAT activity in rats has been shown to be induced by the in vivo administration of a variety of mammalian hormones. Thus, the adult rat liver enzyme responds to hydrocortisone (Lin and Knox, 1957; Csányi et al., 1967), insulin (Holten and Kenney,

1967) and glucagon (Civen et al., 1967; Csányi et al., 1967; Holten and Kenny, 1967; Greengard and Dewey, 1967) and the foetal and post-natal rat liver enzyme to glucocorticoids (Sereni et al., 1959; Yeung et al., 1967; Holt and Oliver, 1969a), glucagon (Civen et al., 1967; Csányi et al., 1967; Holten and Kenney, 1967; Greengard and Dewey, 1967) and the foetal and post-natal rat liver enzyme to glucocorticoids (Sereni et al., 1959; Yeung et al., 1967; Holt and Oliver, 1969a) and insulin and adrenalin (Greengard, 1969; Holt and Oliver, 1969a). The rat liver enzyme can also be induced by cyclic 3',5'-adenosine monophosphate (Greengard, 1969; Holt and Oliver, 1969a).

The diversity of the inducing agents and the observations of a number of anomalous effects inconsistent with a unitary mechanism of induction prompted the suggestion that the enzyme may exist as a number of multiple molecular forms which respond independently to different hormonal stimuli (Holt and Oliver, 1968, 1969a). Previously, electrophoretic and chromatographic analyses of the purified enzyme had revealed the presence of two or more components of varying enzymatic activity (Canellakis and Cohen, 1957; Hayashi et al., 1967; Kenney, 1962; Granner et al., 1968; Tryfiates, 1969; Valeriote et al., 1969). Since then, experiments on the crude enzyme have confirmed the earlier results (Holt and Oliver, 1969a,b, 1971; Sadleir et al., 1970; Iwasaki and Pitot, 1971; Blake and Broner, 1970). The isozymic pattern of activity was also shown to respond to the administration of a variety of substances in the intact animal. These included the hormones hydrocortisone, glucagon, epinephinine, insulin and adrenalin and compounds such as pyridoxine and cyclic 3',5'-adenosine monophosphate (Holt and Oliver, 1969, 1971; Iwasaki and Pitot, 1971).

MATERIALS AND METHODS

1. Time-dependent Variations of TAT Activity

The immature development of insects is characterized by a series of moults, each preceded by a period of dissolution of the dermal layers, followed by its reconstruction and the subsequent sclerotization of the newly-formed cuticle. Accordingly, the metabolism of tyrosine, which serves as the precursor for the formation of the tanning substrates, varies in its response to the changing biochemical requirements during the insect's growth and development. Thus, tyrosine metabolism alternates between constructive and degradative pathways in the moult and intermoult periods, respectively. The metabolic shift within the moult cycle has been detected in a number of insects: Calliphora (Karlson and Sekeris, 1962b; Sekeris and Karlson, 1962), Schistocerca (Karlson and Herrlich, 1965) and Periplaneta (Hopkins et al., 1971) and is qualitatively the same whether encountered in the larva to pupa moult of holometabolous insect or the nymph to nymph moult of a hemimetabolous insect.

Of concern in the present investigation is the enzyme catalyzing the transamination reaction between tyrosine and α-ketoglutaric acid to form para-hydroxyphenylpyruvic acid and glutamic acid, that is, tyrosine-2-oxoglutarate transaminase or TAT. There is evidence that, in insects as in the mammals, the transamination reaction is the first step in the degradative metabolism of tyrosine although the subsequent reactions are different and less is known about the fate of their end products (Karlson

and Sekeris, 1962b; Karlson and Herrlich, 1965; Mills et al., 1967; Mills and Lake, 1971). The shift in tyrosine metabolism is indicated by the decreased production of para-hydroxyphenylpyruvic acid, suggesting a drop in TAT activity, towards the end of an instar. Concomitantly, the production of N-acetyldopamine and other tanning substrates increases.

In this section, the variation of TAT activity throughout the development of a mosquito, Aedes aegypti (L,) was investigated to see how it compared with that of the insects so far studied. An attempt was also made to detect a duirnal rhythm of activity in the mosquito enzyme, a phenomenon known to be exhibited by the rat liver enzyme (Wurtman, 1968; Wurtman and Axelrod, 1967; Civen et al., 1967; Shambaugh et al., 1967).

(i) Insect Culture

Stock of Aedes aegypti (L.) used in this study was obtained from the Institute of Medical Research (IMR), Kuala Lumpur. All adult insects were maintained in cages of dimensions 45 x 45 x 45 cm under normal tropical conditions of temperature 30 $\pm$ 2°C, relative humidity 70%. Sucrose solution, of strength 10-20% (w/v), soaked in a cotton-wool pad, and raisins, previously plumped in distilled water, were offered as food. These food items were changed every 5 days or as often as necessary to prevent fungal growth.

The female mosquitoes were given access to blood (guinea pig for 3-4 hours at a stretch) four days after emergence and then, regularly at four-day intervals after the initial bloodmeal. Eggs were laid about 4-7 days after

a bloodmeal, on a filter paper cone inverted over a glass Petri-dish of distilled water. The oviposition paper was collected and changed once a week and stored in a plastic screw-capped container after a 2-day moisture conditioning period. The conditioning period was necessary to increase the ability of the eggs to resist dessication (Christophers, 1960). All eggs were used within a month of collection.

In those experiments requiring adult mosquitoes of known age to be used, newly-emerged insects were etherized, sexed and placed in 1000 ml glass or plastic beakers covered with a piece of muslin. About 50 individuals were accommodated in each beaker and plumped raisins were offered as energy source. After 3 of 7 days, the insects were sacrificed. Rearing conditions were similar to those of the caged individuals.

To obtain larvae of uniform age, the eggs were vacuum-hatched (15-20 mm Hg) for 30-45 minutes. The newly-hatched larvae were transfered to plastic rearing pans (33 x 25 x 14 cm), each containing about 3 litres of water. The larvae were fed daily on a diet of brewer's yeast or a mixture of ground dog biscuit, yeast extract or powdered dried yeast and ground dried beef liver in the proportion of 10:4:1 or 3:1:1 by weight. The amount of food given varied with the stage of development and the abundace of larvae in the rearing pans. Care was taken that the rearing pans were not overcrowded. The larvae and pupae were reared at 29 $\pm$ 2^{o}C (air temperature), on a lighting regime of 12 hours light (06^{00} to 18^{00} hours)

alternating with 12 hours of darkness (18^{00} to 06^{00} hours) in an environmental chamber.

(ii) Insect Age Terminology

The age of the insects was numbered sequentially in days from day 0, the time the eggs were put to hatch. Thus, by day x was meant the same time on the day following any operation.

There were four larval instars, each of the first three instars lasting approximately the same length of time (about 18-24 hours) with the final instar extending over 52-60 hours. The larval stages took about 4½ days to complete and the pupal stage just under another 2 days, when reared under the conditions described in Insect Culture.

The fourth instar was further subdivided into seven successive stages according to the progressive changes in the size and shape of the presumptive adult eyes which first appeared as a pair of pigmented linear thickenings of the larval epidermis (Spielman and Skaff, 1967). The seven stages and the accompanying forms of the presumptive adult eyes were:

1) IVB_1 - Adult eyes just visible as very fine pigmented strips; head capsule very lightly tanned.

2) IVB_2 - Adult eyes linear, each consisting of 2 definite, unjoined strips; head capsule tanned.

3) IVC_1 - Adult eyes taking on crescentic appearance due to union of the 2 strips but 2-part structure still apparent, i.e., crescent is imperfect.

4) IVC_2 - Adult eyes fully crescentic.

5) IVD_1 - Apex of crescent on ventral side of head begins to broaden and become truncated.

6) IVD_2 - Apex is fully truncated and appears "squared-off" on ventral side of head while on dorsal side, the other apex is well-developed and almost reaches back of head. Pupal air trumpets, if present, untanned and therefore, not visible.

7) Pharate pupa - Air trumpets very apparent due to appearance of pigmentation.

IVB stage was of the longest duration, lasting over 21 hours. IVC and IVD stages lasted approximately 15-17 hours each and the briefest stage, the pharate pupa, about 1½-2 hours (Sengupta, unpublished data).

The pupae, as they emerged on the fifth and subsequent

days, were separated from the larvae and sexually differentiated on the basis of differences in size and character of external genitalia (Christophers, 1960). A portion of the sexed pupae were sacrificed for enzyme preparation while the remainder were put in small plastic bowls and replaced in the environmental chamber for adult emergence.

(iii) Enzyme Extract Preparation and Assay

The collection of the insect material and preparation of their homogenates were carried out at approximately the same time every day (at about 15^{00} hours) so as to obviate any time-dependent variations of enzymatic activity, such as a diurnal rhythm (see under "Diurnal Variation of TAT Activity").

a) Homogenization. Counted samples of larvae and pupae were thoroughly rinsed in distilled water and placed on tissue paper to remove excess moisture. The samples were then weighed to determine wet weight and transfered to an ice-cold manual glass homogenizer of 5 ml capacity. 10 ml of chilled 0.25M sucrose (at about $5^{o}C$) was added to every gram wet weight of insect tissue. Adult mosquitoes were lightly etherized, counted, weighed and homogenized as above. Dead specimens were discarded.

b) Centrifugation. For routine procedures, the homogenates were centrifuged at 1,500 g for 15 minutes

in a bench centrifuge. The supernatant fraction obtained was stored in a low temperature incubator at -20°C. Preliminary experiments indicated that the enzymatic activity of the preparation was unchanged by freezing even after 12 weeks of storage. Hence the studies were conducted on samples of the frozen homogenate within one month of preparation.

c) Enzyme assay. The enzyme assay used was a modification of that described by Sereni et al. (1959). The reaction mixture of 2.5 ml final volume contained 8.4 μM L-tyrosine, 0.168 μM pyridoxal phosphate, 42 μM of phosphate buffer (pH 7.4), 700 μM sucrose, 7 μM diethyldithiocarbamate (DIECA) and 0.2 ml of the enzyme preparation. After a pre-incubation period of 10 minutes, the reaction was initiated by the addition of 30 μM ∝-ketoglutarate, and allowed to proceed for 20 minutes. All reactions were run in a constant-temperature water bath held at 37 ± 0.5°C. Deproteinization was carried out after the allotted time by the addition of 0.5 ml of an aqueous solution of 30% (w/v) trichloroacetic acid. The reaction tubes were then immediately held in ice for 10 minutes, followed by centrifugation for 15 minutes to remove denatured protein. A "zero-time" control was prepared in which trichloroacetic acid was added to the reaction mixture prior to the