GLUCONEOGENESIS IN VITRO IN VESPA ORIENTALIS HAEMOLYMPH.

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INTRODUCTION

In previous papers (Fischl and Ishay, 1971; Fischl et al., in press), we described the high dynamic carbohydrate turnover in the haemolymph and other body tissues of Vespa orientalis (Hymenoptera). Preliminarily the liberation of various sugars and, in some cases, the appearance of extremely large amounts of glucose, was attributed to sacchatrolytic activity only. However, the glucogenesis was so extensive that we suspected that not only the carbohydrate reserves but also some other chemical groups such as amino acids may be mobilized. The present study was undertaken in order to ascertain whether the haemolymph of wasp larvae is able to synthesize glucose in vitro, in a cell-free system.

MATERIALS AND METHODS

Haemolymph from hornet larvae was aspirated under aseptic conditions, at $0^{o}C$, by puncturing the body wall. The haemolymph from numerous larvae was pooled, then frozen and stored at $-20^{o}C$. For determinations requiring incubation with larval midgut, this organ was surgically removed and was frozen before use. In every instance, the test material was first centrifuged at X 5000 g for 20 minutes and the supernate only was used in the various determinations. Glucose determination, as based on glucose oxidase, was done according to the method of Kingsley and Getchell (1960). Sterility of the haemolymph was tested on agar blood plates (Joshua et al., in press). The radiological determinations were carried out as follows: To 16 ml of pooled haemolymph stored at $-20^{o}C$ for two years, C^{14} labelled glutamic acid (4.5 mg)* of 0.1 mC activity was added, and the pH adjusted to 6.5. The mixture was then divided into three equal samples. Sample No. 1 was deproteinized after 15 minutes, by alcohol, and the amino acids and other water soluble components concentrated by the method of Fischl and Segal (1963).

Samples Nos. 2 and 3 were incubated at $37^{o}C$ for 120 minutes, the former alone but the latter together with 500 mg of midgut tissue and contents (which had been previously removed from larvae and frozen at $-20^{o}C$). After incubation, these two samples were also treated as described for Sample No. 1, and aliquots of all three were electrophoresed at 700 v for 1.5 h in formic-acetic acid buffer, at pH 2.2. The electropherograms were scanned by the Dunschiecht Scanner

* DL-Glutamic-1-C^{14} New England Nuclear Corp.

Autochron, Model II, Berthold, at a scanning speed of 1200 mm/h at a time constant of 10 and at a measurement sensitivity range (M.R) of 10 and 30. For a blank (B) we used an aliquot of the labelled glutamic acid containing the same amount of C^{14} as the electropherograms under U.V. illumination and marking the spots by pencil. Amino acids were stained by ninhydrin (0.1% in acetone). Hydrolysis was performed by boiling the samples for 3 hours in a 1 NHCl solution in a water bath.

RESULTS

In the scan, no other amino acid except glutamic acid was found to possess radioactivity. Two fluorescent spots were detectable on each electropherogram. The one nearest to glutamic acid proved to be glucose but the other, about equidistant from the former, is as yet unidentified. The uptake of C^{14} by the glucose begins shortly after the start of incubation: in Sample No. 1 only one significant peak is observable (next to the glutamic acid) but in samples Nos. 2 and 3 two significant uptake peaks occur (Fig. 1). Quantitatively greater amounts of radioactivity are detected in Sample No. 3 than in No. 2. This difference in radioactivity is difficult to discern at the higher sensitivity level (MR:10) but is far more evident in the scans taken at the lower sensitivity level (MR:30) where the labelled glutamic acid shows a striking decrease from sample to sample 3. (Fig. 2).

The purity of the labelling material is shown in Fig. 3. In Fig. 4 the scans are superimposed over the paper chromatograms of the samples and the C^{14} uptake by the glucose is thereby verified.

The glucose levels in the haemolymph during the incubation at 37^{o}, pH6, are presented in Table 1, where the results are given as mean mean values based on three determinations each.

Table No. 1 Glucose Levels (mg% in Haemolymph).

Incubation Time	Haemolymph	Haemolymph with midgut
0	475	474
15'	545	2450
30'	660	3270
60'	820	3880
120'	945	3960

Samples of unincubated haemolymph when hydrolyzed in 1NHCl for 3 hours, gave a total glucose reading of 3200 mg%. Similar hydrolysis of midgut incubated haemolymph did not alter the previous mentioned glucose level (i.e. 3960). The results were reproducible in two additional experiments. In none of these experiments was glucose detected in the midgut, not even after incubating the midgut in 0.85% saline for 2 hours. Haemolymph alone (Sample No.2) darkened when allowed to stand overnight at room temperature, but haemolymph

in which midgut was immersed (Sample No. 3) did not (Fig. 5).

DISCUSSION

The experiments described above prove that gluconeogenesis does occur in the cell-free haemolymph of the hornet larva. The reaction is accelerated in the presence of midgut and the difference between the results with and without midgut is possibly even greater because the activity of monosaccharidases was not assessed.

That at least one, if not the only, source of material for the glucose may be amino acids was demonstrated by the experiment with C^{14} glutamic acid. We found, however, that not only glucose is generated but also some other derivative substance. The latter neither stained with ninhydrin nor responded to amino-sugar detecting agents, so it cannot be an amino compound. The possibility that it may be trehalose was also discounted when its incubation with trehalase failed to produce glucose. It would seem that our 'unknown' is not a condensation but rather a break-down product of glucose and our search is continuing in this direction.

The fact that complete hydrolysis of the carbohydrates in the haemolymph yielded less glucose than incubation of the haemolymph with mid-gut (which contains no glucose and little or no other saccharides - Fischl et al, in press), indicates that the complex of haemolymph mid-gut contains some biological substances which are capable of generating glucose from non-carbohydrate sources.

Indirect evidence of gluconeogenesis is offered by the fact that haemolymph, in contact with mid gut, retains its original straw colour whereas haemolymph alone darkens upon standing.

The darkening upon standing of insect haemolymph is a well known phenomenon (Brunet, 1965) which resembles mammalian melanogenesis or the darkening of homogentisic acid in the absence of homogentisate (Lerner, 1953). Aromatic amino acids, which are the natural precursors of melanin, are abundant in the hornet larva haemolymph (Ikan and Ishay, 1973; Joshua et al., in press). In the case of accelerated gluconeogenesis, as caused by some factor(s) in the mid-gut, most of the tyrosine was probably directed towards gluconeogenesis via the citric acid cycle at the side of the formic acid (Fig. 6).

In addition, however, there may be some factors in the mid-gut which lead to detoxification reactions, whereby phenols and their derivatives, such as the precursors of melanin, are converted into the corresponding β-D-glucosides. Such reactions occur normally in the insect fat body (Hines and Smith, 1963). In mammals, phenols are conjugated as glucosiduronides while in insects they appear as β-D-glucosides. It seems to us that the acceleration of glucose formation by mid-gut may serve the latter purpose, that is to say, if more glucose is available, there is a greater potential for conjugation of phenol compounds, which implies enhanced detoxification. In the conjugated form the phenols are not subjected to oxidation by phenolase (Brunet, 1965). It may be reasonable that such an accelerated

ability of detoxification is present in the digestive tract of the larvae. In the larvae of Vespa, as most Apocrita, the mid gut is a blind sac and does not communicate with the hind gut until the final instar, the faecal contents only being evacuated at the conclusion of the larval stage (Imms, 1960).

In our in vitro studies, we found that the increment in glucose which occurs in the course of incubation with mid-gut is in the vicinity of 20%. In other words, the glucose level is higher by about 20% than that in haemolymph which had undergone acid hydrolysis, a process whereby glucose is released from all available carbohydrate sources. However, despite the considerably lower glucose levels in haemolymph not incubated with mid-gut, the experiments with the labelled glutamic acid clearly show the formation of glucose from it even in the absence of mid-gut. The gluconeogenetic process is probably very rapid, inasmuch as C^{14} glucose is already present after incubation for fifteen minutes with haemolymph alone. Nevertheless, it is clear that the gluconeogenetic process is accompanied in all instances by carbohydrate hydrolysis which, as is evident from the experimental results, is the primary cause for the large increase in the in vitro glucose level of haemolymph.

All the experiments prominently display the incorporation of C^{14} into glucose as well as into the as yet unidentified derivative substance. It is quite surprising, however, that in no instance was there incorporation of C^{14} into products of smaller molecular size such as other amino acids. Does this imply that the gluconeogenetic process dominates in our system to such an extent that it inhibits all other incorporation processes, or rather simply that other processes do not occur in vitro?

Also surprising is the high threshold level of glucose in frozen larval haemolymph. The haemolymph of living larvae contains ordinarily 50-150 mg. glucose per 100 cc. On the other hand, in haemolymph that had undergone freezing the glucose level is 5-10 fold higher. A likely explanation for this discrepancy is that the freezing and subsequent thawing somehow releases monosaccharides, possibly by triggering trehalase and other saccharolytic enzymes abundantly present in larval haemolymph (Wyatt, 1967; Fischl et al., in press).

In a previous study (Ishay and Ikan, 1968), we fed Vespa orientalis larvae on a C^{14} labelled protein and shortly after, the larval saliva secreted in the course of food exchange (trophallaxis of Wheeler, 1968), was found to contain considerable amounts of C^{14} glucose and other C^{14} labelled products. We deemed this proof to the occurrence of gluconeogenesis in vivo in hornets. Considering that these insects ordinarily subsist on a diet poor in carbohydrates (they feed only on the flesh of other insects during most of the active season - Ishay et al. 1967), one would expect the energy-yielding gluconeogenetic process to be enhanced in vivo. The present study confirms the occurence of gluconeogenesis in hornets also in vitro. In general outline, the process here conforms to the in vitro gluconeogenesis which Krebs (1964) obtained with pigeon liver homogenate.

A rise in the glucose level of the haemolymph has been reported previously for various insects. Medvedeva (1935, 1939) described hyperglycemia in silkworm caterpillars following the administration of adrenalin, Wyatt (1961, 1963) and Stevenson and Wyatt (1964) observed that when pupae of Hyalophora cecropia receive the injury factor, i.e., when the integument of the diapausing pupa in injured, there is a rise in the blood trehalose level, decrease in the fat-body glycogen content and activation of phosphorylase. Local wounding of the integument causes a rise in the respiratory rate for several days (Harvey and Williams, 1961) but the conversion of C^{14} glucose to CO_2 remains unchanged (Wyatt, 1963). It is clear, however, that wounding also causes a rise in the respiratory rate in isolated abdomens devoid of endocrine glands, so that this rise of glucose is not associated with the activity of any particular gland.

In the present study we encountered in vitro hyperglycemia of haemolymph which became accelerated during the incubation with mid-gut. In general outline, the response may possibly resemble that of wounding: the mobilization of glucose precursors and the synthesis of glucose which is not utilized in respiratory processes. It seems that mid-gut tissue accelerates the process of glucose synthesis in order to provide an acceptor for the formation of β-D-glucosides for the purpose of detoxification. The detoxification process is apparently an immediate, reflexory response, both in the course of injury, as well as in the present case whenever exposure to free air takes place.

SUMMARY

In haemolymph collected aseptically from Vespa orientalis larvae, there is spontaneous formation of glucose. Incubation of such haemolymph with larval mid-gut yields larger amounts of glucose. Incubation of the haemolymph with C^{14} labelled glutamic acid results in the formation of C^{14} labelled glucose, which proves that the rise in the glucose level of the haemolymph is at least partly due to gluconeogenesis.

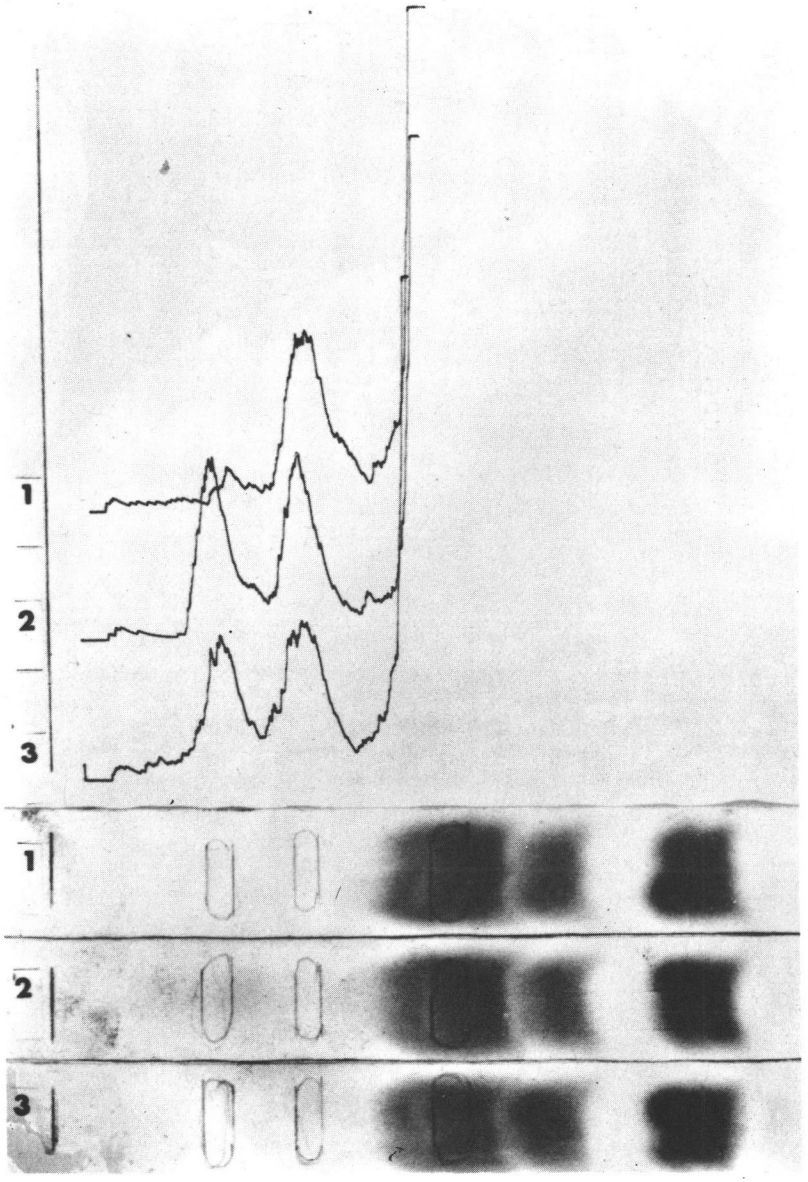


Fig. 1.

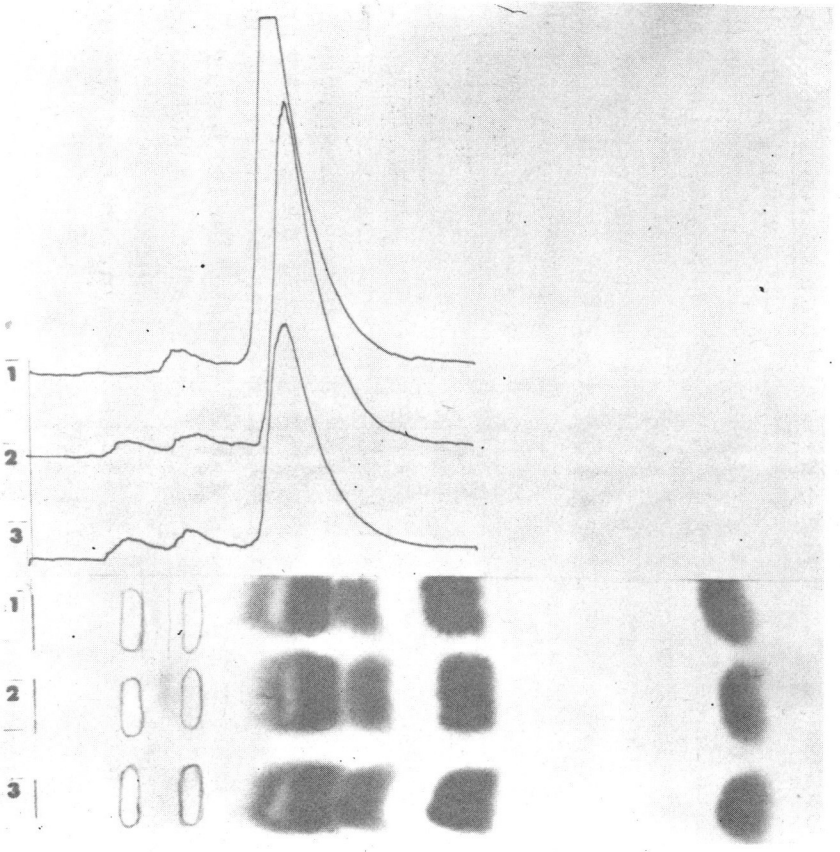


Fig. 2.

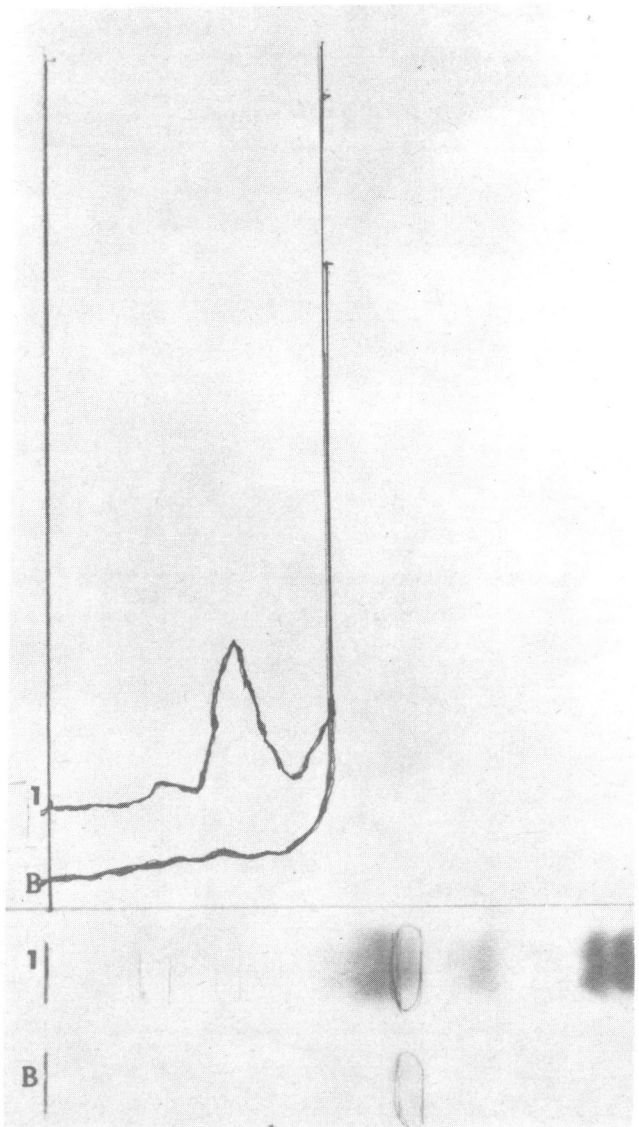


Fig. 3

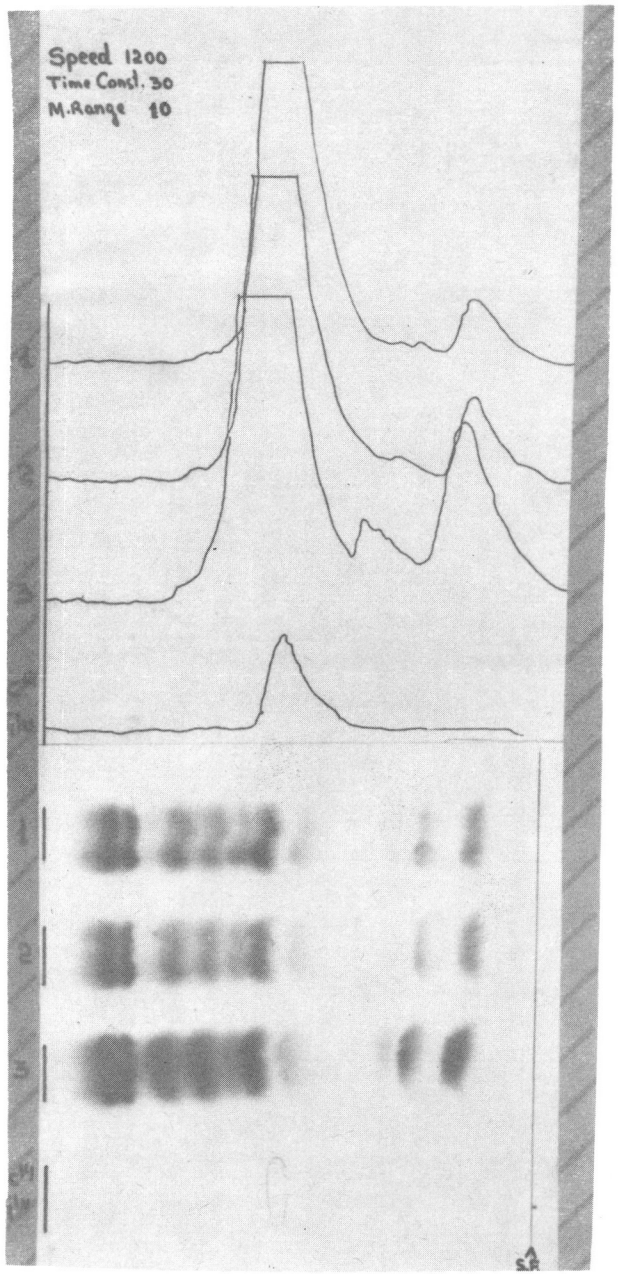


Fig. 4.

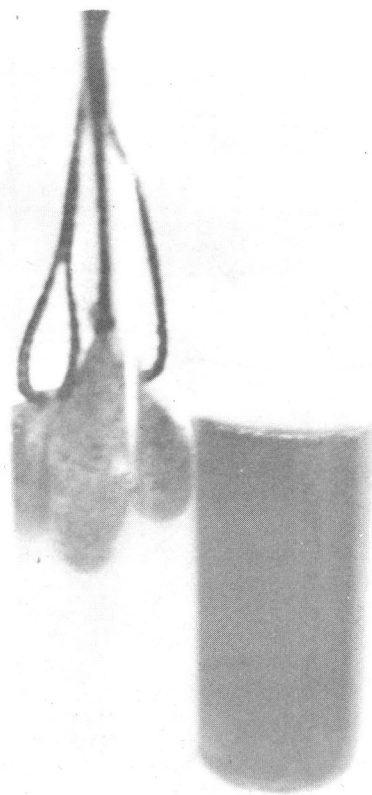
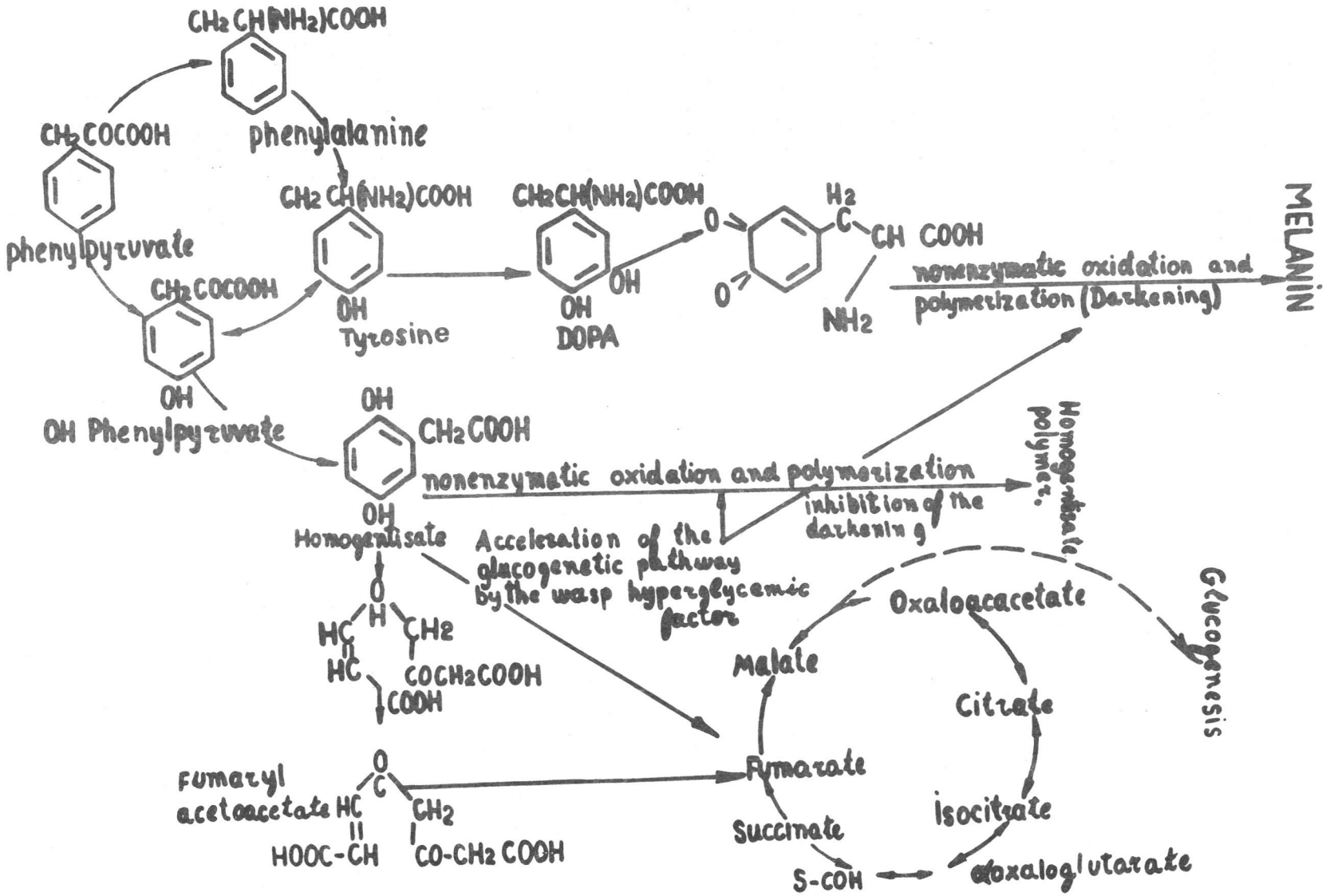


Fig. 5.

Fig. 6 The influence of wasp "hyperglycaemic" factor on the glucogenetic pathway of Tyzosine



LEGENDS TO FIGURES

Fig. 1. Uptake of glutamine C^{14} by haemolymph
1. Haemolymph after addition of labelled glutamic acid about 15 minutes standing.
2. Haemolymph after 1 hour incubation.
3. Haemolymph plus mid-gut after 1 hour incubation. Lower part of the picture shows the ninhydrin staining (dark spots) of amino acids and the fluorescent fractions (pencil marking) of the electropherogram.

Fig. 2. The same as Fig. 1 but sensitivity reduced by a factor of 10.

Fig. 3. Demonstration of absence of peaks in the labelling material B compared to Sample 1 as in Figs. 1 and 2. The absence of other amino acids in the labelled compounds is demonstrated in the lower part of the picture of high voltage electrophoresis.

Fig. 4. Paper chromatography of C^{14} labelled glutamic acid. Samples 1, 2 and 3 and their scannogram.

Fig. 5. Demonstration of inhibition of darkening of the haemolymph by immersion of mid-gut overnight at room temperature.
Left: haemolymph with mid-gut, Right: the same sample without mid-gut.

Fig. 6. The probable metabolic pathway of tyrosine in the presence of mid-gut accelerating gluconeogenesis and thereby reducing polymer formation (melanin-like substance).

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