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Transaminating Processes Involving Histidine in Human Haemolysed Erythrocytes

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Haemolysates of human red blood cells show transaminating activity not only between α-ketoglutaric acid and both alanine and aspartic acid, but with other amino acids not observed previously.

The behaviour of histidine in particular was studied in these reactions since, by incubation with α-ketoglutaric acid, glutamic acid was formed, but with pyruvate aspartic acid was unexpectedly found.

The presence of aspartic acid was demonstrated by paper chromatography in different solvents and by paper electrophoresis. Imidazole derivatives were detected with Pauly's reagent.

A reaction scheme is proposed whereby pyruvate enters partly a transaminating process with histidine, from which the resulting imidazolyl-pyruvate gives imidazolyl-acetate by decarboxylation.

The carbon dioxide released in this process reacts with pyruvate in a Wood-Werkmann reaction, giving rise to oxaloacetate, from which aspartic acid is finally formed by a secondary amino-transfering reaction with surplus histidine.

Imidazole acetate was detected by paper chromatography, and imidazole pyruvate by the enol-borate tautomerase method.

According to earlier investigations, mature human erythrocytes (red blood cells = RBC) contain a very active glutamic-oxaloacetic transaminase (GOT).

Merten and Ess[1] found 126,000 ± 17,000 W. U.** in 10^{12} RBC. The activity of glutamic-pyruvic transaminase (GPT) was found to be far lower, and transamination with other amino acids failed to be established.

In 23 haemolysates of human RBC we found 1.233 ± 278 W. U. of GOT in 10^{10} RBC,*** in agreement with published data; moreover the mean activity for GPT was only 171 ± 55 W. U. in 10^{10} RBC.

We noticed, contrary to expectations that some other added amino acids entered transaminating processes with both α-ketoglutaric and pyruvic acids, the former usually exhibitting a much higher activity.[2]

In studing transaminating phenomena with different amino acids, our interests were drawn to the behaviour of histidine. This amino acid should be of importance in RBC metabolism owing to its high concentration in haemoglobin, and to the role of its imidazole ring in binding globin to haem.

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** Wróblewski units

*** 10^{10} RBC equal to 1 ml. packed cells.

Histidine entering an in amino group exchange with α-ketoglutaric acid gave rise to glutamic acid, as could be expected. With pyruvic acid however, it behaves quite unusually and this is the subject of the present paper.

EXPERIMENTAL

The haemolysates were prepared from oxalated blood. The isolated RBC were washed three times with physiological saline. After each washing the sample was centrifuged and the supernatant removed, the top layer wiped off with a cotton plug and the RBC sediment transfered with a pipette into another test tube, taking the sample only from the middle to avoid the leucocytes on the tube wall. Using this precaution the RBC were practically free from leucocytes. The RBC were then diluted 1 : 5 with distilled water and completely haemolysed by freezing twice at 0^0 C for 30 minutes. The haemolysate was ready for transamination experiments after filtering off stroma particles.

The following solution were prepared in 0.1 *M* phosphate buffer, *p*H 7.4:

L-histidine hydrochloride 0.1 *M*	*BDH*
α-ketoglutaric acid 0.1 *M*	*BDH*
pyruvic acid 0.1 *M*	*BDH*
oxaloacetic acid 0.1 *M*	*BDH*

Procedure: 0.5 ml. haemolysate, 0.5 ml. of the corresponding keto acid, and 0.5 ml. histidine solution were pipetted into a centrifuge tube, filled up to 3 ml. with buffer and incubated for 3 hours at 37^0 C. The enzymatic process was stopped by the addition of 10 ml. of absolute ethanol .After 10 minutes the reaction mixture was centrifuged, and the clear supernatant decanted into a wider test tube. The precipitate was washed twice with 70% ethanol, the washings united and added to the supernatant. The alcohol was evaporated on a water-bath and the residue dissolved in 0.5 ml. of phosphate buffer. The resulting amino acids were identified by paper chromatography and paper electrophoresis.

Ascending chromatography was performed according to an earlier report[3] on *Whatman* paper No. I with phenol: water (4 : 1),[4] pyridine: acetic acid: water (50 : 35 : 15),[5] and *n*-propanol: acetic acid (3 : 1).[6] The chromatograms were air-dried for 24 hours, and when the solvent contained phenol, they were held for an additional hour at 70^0 C. The dry chromatograms were sprayed with 0.5% ninhydrin solution in acetone : propanol (1 : 1) left for 2 hours at room temperature and finally heated for 20 minutes at 60—70^0 C. The spots were fixed by spraying with a solution of copper nitrate ,and air dried.[7]

Paper electrophoresis on *Whatman* No. 1 strips was performed for 4^h with 10 V/cm. in a pyridine : acetic acid : water buffer (6 : 30 : 964) *p*H 3.9.[8]

For the identification of the amino acids and imidazole derivatives formed in the reaction mentioned above, a combination of electrophoresis and chromatography was found very convenient, performing the former in one direction, and the latter in the other.

Pauly's diazo reagent was used for the detection of imidazole derivatives: a mixture of equal parts of a 4.5% solution of $NaNO_2$ and a 0.9% solution of sulphanilic acid in N-HCl was kept for half an hour in ice and mixed with two parts of 10% sodium carbonate immediately before spraying.[9]

The presence of imidazole pyruvic acid was demonstrated by the enol-borate tautomerase method.[10]

RESULTS

The incubation of a substrate consisting of histidine and α-ketoglutaric acid with the haemolysate resulted in the appearance of glutamic acid, as shown in Fig. 1, the degree of transfer being about one fifth of that shown by RBC-GOT.

If histidine and pyruvic acid were added as substrates to the haemolysate, not only alanine is found but also, unexpectedly, is aspartic acid (Figs. 1

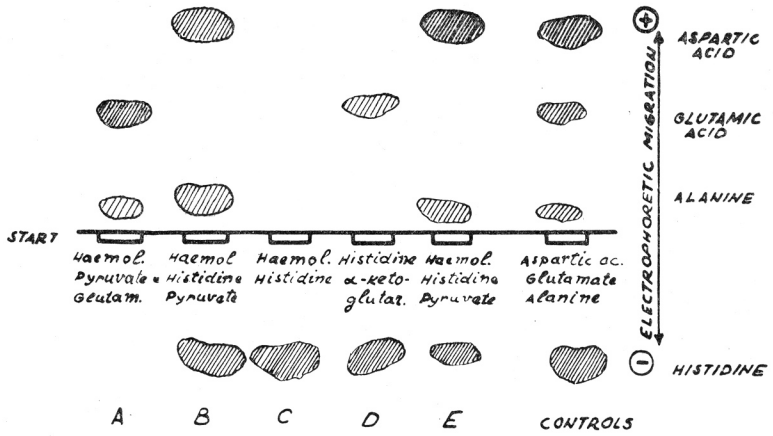

Fig. 1. Electrophoretic patterns of transamination reactions: A- under GPT influence alanine is formed, B- and E- by transamination reactions of histidine and pyruvic acid, alanine and aspartic acid are formed. D- transamination of histidine and α-ketoglutaric acid.

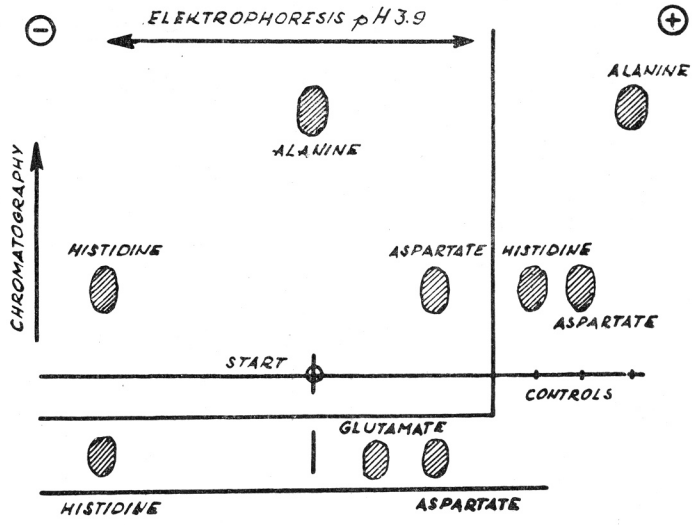

Fig. 2. Combined electrophoresis and chromatography of transamination products between histidine and pyruvate. On the right are chromatographied controls, and on the base the electropherogram of the controls. The tvo-way analysis shows the resulting aspartic acid and alanine.

and 2), and in the case when both pyruvic acid and α-ketoglutaric acid are added together with histidine, alanine, aspartic acid and glutamic acid were found.

Aspartic acid was identified by chromatography in different solvents and by paper electrophoresis. It was also found when oxaloacetic acid was used as substrate instead of pyruvic acid (Fig. 3). All of these reactions are more pronounced in the presence of 0.1 ml. of 0.1 *M* pyridoxal phosphate as shown in Fig. 3.

If the haemolysate was added to a solution containing histidine and oxaloacetic acid, aspartic acid was formed by transamination. In replacing histidine with alanine, transamination did not take place even in the presence of pyridoxal phosphate (Fig. 3). These findings indicate that histidine, not the alanine produced, figures as the amino-group donor for oxaloacetic acid in our experiments.

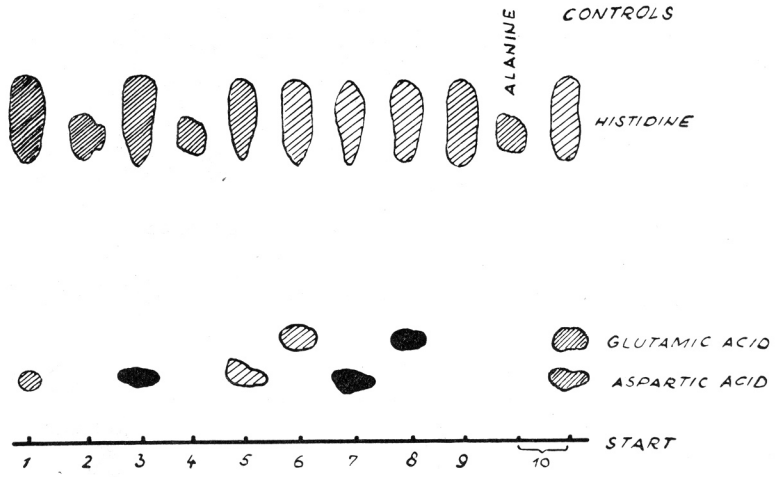

Fig. 3. Demonstration of pyridoxal phosphate influence on the transaminating action of the haemolysate: 1. Histidine and oxaloacetic acid; 2. Alanine and oxaloacetic acid; 3. Histidine and oxaloacetic acid with pyridoxal phosphate added; 4. Alanine and oxaloacetic acid with pyridoxal phosphate added; 5. Histidine and pyruvic acid; 6. Histidine and α-ketoglutaric acid; 7. Histidine and pyruvic acid with pyridoxal phosphate added; 8. Histidine and α-ketoglutaric acid with pyridoxal phosphate added; 9. Histidine; 10. Histidine, alanine glutamic and aspartic acids as controls: Paper: Whatman No. 1; Solvent: phenol : water (4 : 1).

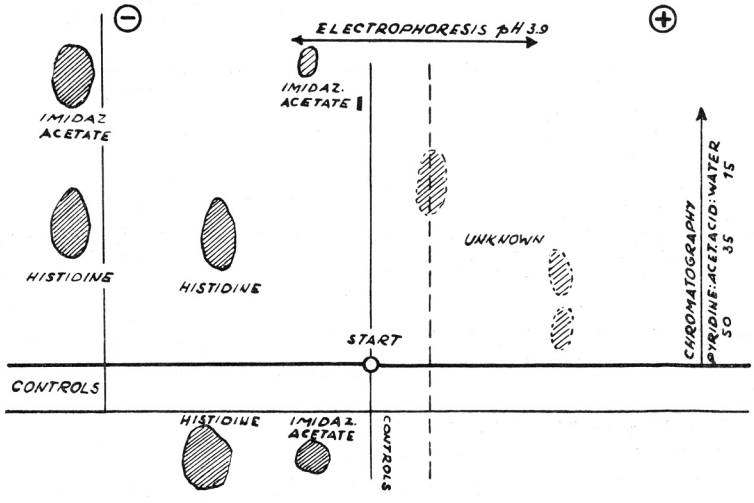

Fig. 4. Electrophoresis and chromatography of transamination products of histidine with pyruvic acid. On the left are the controls: imidazole-acetate and histidine. On the base the same ones, cut off after electrophoresis. The spots were detected with Pauly's diazo reagent.

When the chromatograms and electropherograms of the haemolysate, after incubation with histidine and pyruvic acid, were sprayed with Pauly's diazo reagent, imidazole acetic acid could be detected (Fig. 4) and some other spots representing probably degradation products of the imidazole pyruvic acid were observed. Treating the same sample by the enol-borate tautomerase method[10] an adsorption maximum was obtained at 293 mμ indicating the presence of imidazole pyruvic acid.

DISCUSSION

The formation of aspartic acid in transamination reactions between histidine and pyruvic acid, with the human RBC haemolysate as enzyme source, is in fair agreement with recent findings on the metabolic fate of histidine. Baldridge *et al.*[11] and Wolf *et al.*[12] found imidazole acetic acid in the urine of histidine-fed animals. All of these authors suppose that the imidazole acetic acid was formed via imidazole pyruvic acid. We assume that in our experiments the resulting aspartic acid could only be formed by transamination from oxaloacetic acid which must have originated in the course of these reactions. The fact that the same results were obtained when pyruvic acid was replaced by oxaloacetic acid as substrate confirm this statement. It is true that Hayaishi *et al.*[13,14] found that histidine gave rise to imidazole acetic acid in extracts of Pseudomonas, which, in the presence of $NADH_2$ and a molecule of oxygen resulted in the formation of aspartic acid *via* formimino-aspartic acid and formyl-aspartic acid. In our case, however, this method of formation of aspartic acid is not possible since it would also be formed without the addition of pyruvic acid.

The enzymatic CO_2-fixation reaction leading from pyruvic acid to C_4-dicarboxylic acids has been referred to as the »Wood-Werkmann reaction«. Earlier work in this field has been fully reviewed by Werkmann and Wood[15] and Utter and Wood.[16] Ochoa *et al.*[17] have demonstrated the presence of malic enzyme which catalyzes the formation of malic acid from pyruvic acid and CO_2. Malic acid formed in this reaction is oxidized to oxaloacetic acid by NADP in the presence of malic acid dehydrogenase. A second CO_2-fixation reaction, found by Utter *et all.*[18,19] is catalyzed by phosphoenolpyruvic acid carboxylase. This reaction requires IDP. Utter has recently described a third enzyme, pyruvic acid carboxylase, which catalyzes the formation of oxaloacetic acid from pyruvic acid and CO_2. Several aspects of this system suggest that it may also be the most significant single device for *de novo* formation of oxaloacetic acid.[20] From experiments on rat liver it is known further that pyruvic acid can enter the tricarboxylic acid cycle by condensing with carbon dioxide and forming a dicarboxylic acid.[21] In Staphylococcus aureus, carbon dioxide is found fixed in aspartic acid.[22] Finally, fixation of carbon dioxide in oxaloacetic acid is found *in vitro* and in duck RBC.[23,24]

In the light of these facts concerning the fixation of carbon dioxide with pyruvic acid in oxaloacetic acid, and the results obtained from our experiments, we can conclude that the formation of aspartic acid in the reaction of RBC haemolysate with histidine and pyruvic acid is a result of several coupled reactions. The products of this transamination are alanine and imidazole pyruvic acid. The latter of these becomes decarboxylated to imidazole-acetic acid and the liberated carbon dioxide fixed with pyruvic acid, added as sub-

strate, to form oxaloacetic acid. The oxaloacetic acid immediately enters a new transaminating reaction with excess histidine and gives rise to aspartic acid (Fig. 5).

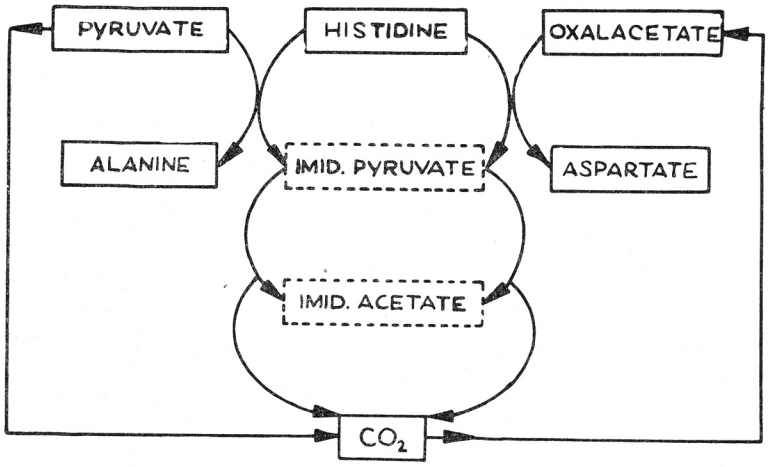

Fig. 5. Reaction scheme.

All these reactions are shifted in the sence of aspartic acid formation. Excess pyruvic acid picks up and eliminates the carbon dioxide generated, and the oxaloacetic acid so formed disappears from reaction mixture by a transamination reaction with histidine. In this manner the whole reaction sequence favours oxaloacetic acid formation and a consecutive transamination to aspartic acid. In the case of histidine transamination with α-ketoglutaric acid, there is no acceptor for the carbon dioxide and the reaction is stopped at the imidazole-pyruvic acid stage. At the moment, we are attempting to verify these results by further investigations.

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IZVOD

Procesi transaminacije s histidinom u hemolizatima čovječjih eritrocita

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Osim transaminacije između α-ketoglutarne kiseline i alanina odnosno asparaginske kiseline, hemolizati čovječjih eritrocita pokazuju transaminirajuću aktivnost i sa drugim amino kiselinama.

Posebno je promatrano ponašanje histidina u tim reakcijama. Transaminacijom s α-ketoglutarnom kiselinom stvara se glutaminska kiselina, dok s pirogrožđanom kiselinom pored alanina nastaje i asparaginska kiselina. Nastala asparaginska kiselina dokazana je kromatografijom na filtar papiru u raznim otapalima te elektroforezom, a imidazolni derivati istim tehnikama i bojenjem Pauly-evim reagensom.

Predložena je shema reakcija po kojoj transaminacijom između histidina i pirogrožđane kiseline nastaju alanin i imidazolil-pirogrožđana kiselina. Dekarboksilacijom ovog potonjeg nastaje imidazolil octena kiselina, a oslobođeni CO_2 sa pirogrožđanom kiselinom stvara oksaloctenu kiselinu iz koje pak sa histidinom ponovnom transaminacijom nastaje asparaginska kiselina.

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