A cautionary note on pentacyanoammonioferrate use for determining L-canavanine occurrence in biological materials[1]

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Summary. Caution must be taken in the prevelant use of the pentacyanoammonioferrate reagent for the detection of L-canavanine in biological materials. This reagent reacts with L-histidine at neutral pH to form a pentacyanoammonioferrate-histidine complex that is mistaken readily for its canavanine-containing counterpart.

Detection of an unexpected amino acid in certain processed meat products was taken recently to be a reliable indicator of adulteration by soya bean, *Glycine max*[2]. This marker compound was identified as L-canavanine, a nonprotein amino acid of leguminous plants[3], since it co-chromatographed by automated amino acid analysis with authentic canavanine and responded to the PCAF (pentacyanoammonioferrate) reagent[2]. This reagent has served as the standard canavanine indicator for a host of chemotaxonomic and metabolic investigations[4-8]. These findings instigated a further study of *G. max* by Rosenthal[9] which confirmed the reported observations but established that the unknown marker amino acid was not canavanine. This example is but one of several known instances of erroneous assertion of canavanine's occurrence in various higher plants and fungi[10,11]. Recent colorimetric analyses of various cultivars of alfalfa, *Medicago sativa* possessing differential resistance to the alfalfa weevil, *Hypera postica* and the potato leafhopper, *Empoasea fabae*, disclosed the presence of a PCAF-positive compound that was taken on first consideration to be canavanine. Since the concentration of the PCAF-positive compound appeared to correlate positively with plant resistance to these insect pests, proper effort was made to elucidate its identity.

Methods and materials. Single dimensional, thin-layer chromatography was conducted with precoated cellulose F TLC plates obtained from E. Merck, Inc. The solvent system consisted of methanol: chloroform: 17% NH_3 (2:2:1, v/v). The observed R_f-value for arginine, homoarginine, canavanine, and histidine were 0.30, 0.35, 0.38, and 0.48, respectively. Canavanine and histidine were detected by spraying with a 1:1 (v/v) mixture of 1% (w/v) PCAF and 100 mM phosphate buffer (pH 7.0). The Pauly reagent (reagent 37)[12] gave a yellow-orange color with histidine but failed to produce a discernible chromogen with canavanine, homoarginine, or arginine. The Sakaguchi reagent (reagent 97)[12] yielded a yellow color with histidine but an orange-pink color with canavanine; pink coloration resulted with homoarginine and arginine.

Automated amino acid analyses were conducted with a system designed for physiological samples analysis by Durram Chemical (Dionex Corp.). The process involves the sequential application of 5 buffers that separate completely histidine (column retention time = 227 min), canavanine (238 min), and arginine (246 min). Some automated amino acid analyzers utilize resin and buffer elution systems in which histidine can co-elute with canavanine or emerge very close to this arginine analogue in the column effluent.

Results. Initial TLC analyses coupled with various colorimetric procedures indicated that this PCAF-positive compound was histidine. Subsequent automated amino acid analyses of *M. sativa* revealed that while canavanine was not detected from our plants, histidine was present and represented a predominate constituent of the basic free amino acid fraction.

Examination of the interaction of histidine and PCAF revealed that this protein amino acid does react to form a chromogen that can easily be mistaken for PCAF-canavanine - particularly with dilute solutions of canavanine. The marked similarity in the absorption spectra for these amino acids complexed with PCAF is shown in figure 1. A significant difference between these 2 chromogens is the much more rapid color formation associated with PCAF-canavanine as compared to PCAF-histidine (fig. 2). This is especially evident when PCAF is utilized as a spray reagent for canavanine detection since the canavanine-PCAF spot is visualized immediately while the histidine-PCAF spot only becomes discernible after the PCAF reagent has dried. In addition, as little as 1 µg of histidine can be detected with this reagent. The possibility of confusing canavanine and histidine is intensified by the fact that canavanine can

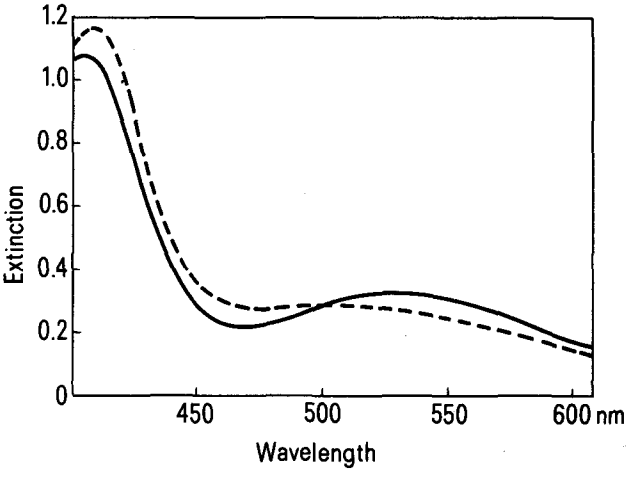

Figure 1. The extinction of the PCAF-amino acid chromogen as a function of wavelength. The PCAF-histidine (--) or PCAF-canavanine (—) complex was prepared with 1.0 mM histidine or 0.25 mM canavanine as previously described[7].

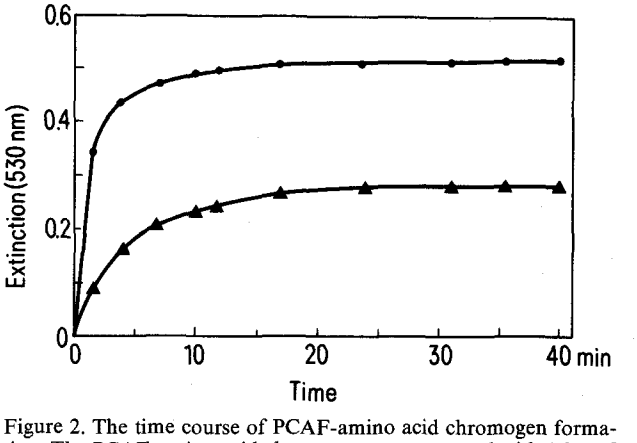

Figure 2. The time course of PCAF-amino acid chromogen formation. The PCAF-amino acid chromogen was prepared with 1.0 mM histidine or (▲) 0.40 mM canavanine (●) as previously described[7].

be separated with considerable purity from complexed biological mixtures with Dowex-50 (NH_4^+) and elution with dilute ammonia[7]. This is the preferred method for the isolation and purification of this arginine analogue. Unfortunately, histidine is one of the very few natural products that co-elutes with canavanine under these experimental conditions. Given the instances of erroneous disclosure of canavanine's natural occurrence and the potentially universal distribution of free histidine, it is evident that assertions on canavanine's occurrence and distribution must not be predicated solely on the use of PCAF or the elution position relative to canavanine of a given natural product.

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Purine nucleotide cycle as a possible anaplerotic process in rat skeletal muscle[1]

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Summary. The intermediates of the purine nucleotide cycle (PNC) stimulated pyruvate oxidation by isolated skeletal muscle mitochondria in a system containing mitochondria and cytosol from rat skeletal muscle. Thus, in skeletal muscle the PNC might be involved in the anaplerotic supply of tricarboxylic acid cycle intermediates.

Three reactions have been suggested which might cause an increase in the content of citric acid cycle intermediates in skeletal muscle[2-6]. These reactions are: a) pryruvate carboxylation catalyzed by pyruvate: carbon dioxide ligase (ADP-forming), (EC 6.4.1.1.)[3,4], b) malate formation catalyzed by extramitochondrial malic enzyme (l-malate: $NADP^+$ oxidoreductase oxaloacetate decarboxylating), (EC 1.1.1.40)[5], c) the reactions of the PNC[2,6]. Some authors have also taken into consideration alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase), (EC 2.6.1.2) and glutamate dehydrogenase (L-glutamate: NAD^+ oxidoreductase deaminating), (EC 1.4.1.2)[2,6]. However, a major role for the last 2 enzymes seems unlikely[6]. The role of pyruvate carboxylase[4] and extramitochondrial malic enzyme[5] in replenishing the Krebs cycle intermediates in skeletal muscle have gained strong support from the recent experiments performed on isolated mitochondria. Aragon and Lowenstein[6] showed that in rat skeletal muscle the total level both of citric acid cycle intermediates and of IMP rises during exercise. They suggest therefore that the PNC is operating in skeletal muscle during exercise and that it might be responsible for an increase of citric acid cycle intermediates.

In this communication, a system is described in which fumarate formed during operation of the purine nucleotide cycle in skeletal muscle cytosol stimulated pyruvate oxidation in isolated muscle mitochondria. The results gave further evidence that the operation of the purine nucleotide cycle may be responsible for the replenishment of citric acid cycle intermediates in skeletal muscle.

Materials and methods. Pyruvate, malate, fumarate, adenylosuccinate, aspartate, GTP, AMP and IMP were from Sigma Chem. Co (USA). All other chemicals were from P.O.Ch. Gliwice (Poland).

Skeletal muscle mitochondria were prepared as described previously[7]. The muscle cytosol was prepared from the hind legs of rats. The muscles were homogenized in 3 volumes of 50 mM phosphate buffer pH 7.0 containing 15 mM KCl and 1 mM dithiothreitol and centrifuged at 20,000 × g for 40 min. The pellet was rehomogenized, and centrifuged again at 20,000 × g for 40 min. The supernatants were combined and centrifuged at 100,000 × g for 40 min. The resulting supernatant was dialyzed overnight against 500 vols of homogenization medium without dithiothreitol. Ammonia was determined according to the method of Seligson and Seligson[8] as modified by Strelkov[9]. After microdiffusion, ammonia was measured colorimetrically by the method of Chaney and Marbach[10]. Oxygen consumption was measured with a Clark electrode under the conditions indicated in the appropriate tables and figures.

Results and discussion. The isolated rat skeletal muscle mitochondria were able to oxidize pyruvate at a low rate in the absence of added malate. When malate was added, the oxygen uptake increased several-fold due to the entry of pyruvate into the citric acid cycle. Fumarate could replace malate in such experiments. Figure 1 demonstrates that in the mitochondrial suspension oxidizing pyruvate, the rate of oxygen uptake increased from a very low rate (about 16 ng-atoms $O \times min^{-1} \times mg^{-1}$) to a maximum rate of about 280 ng-atoms $O \times min^{-1} \times mg^{-1}$ while the concentration of either L-malate or fumarate was increasing. The concentration of L-malate required to reach the maximum rate of oxygen consumption was about 0.5 mM. Similar results were obtained when malate was replaced by fumarate. A possible explanation is that fumarate is taken up into the mitochondria where it is converted to malate by intramitochondrial fumarase. This might be somewhat surprising, because fumarate is claimed to be a substrate that is unable to penetrate into rat liver mitochondria[11]. However, some evidence that fumarate translocation in rat heart mitochondria does take place has been presented recently[12]. It seems likely that this is the case in rat skeletal muscle mitochondria also. However, it is not excluded that the conversion of fumarate to malate is taking place outside the