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Toxicity of Aromatic Acids to the larvae of the Mosquito *Aedes aegypti* L. and the Counteracting Influence of Amino Acids

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Knowledge of the detoxication mechanisms of insects is becoming of ever-increasing importance. The metabolism of insecticides may so alter their biological activity as to destroy their toxicity (Sternburg & Kearns, 1950), produce new poisons in the plant from applied chemicals (Casida, Chapman, Stahmann & Allen, 1954; Metcalf, 1954) or even form the material which is toxic to insects from a non-toxic precursor (Metcalf & March, 1953; Casida & Stahmann, 1953). Locusts have been reported to contain the enzymes for glucuronide and ethereal sulphate hydrolysis (Robinson, Smith & Williams, 1953); they also form hippuric acid from benzoic acid (Friedler & Smith, 1953), glucosides from phenols (Myers & Smith, 1953*a*, 1954) and can acetylate aromatic amines (Myers & Smith, 1953*b*). In addition, insects can dehydrochlorinate certain chlorinated hydrocarbons enzymically (Sternburg, Vinson & Kearns, 1953), and oxidize thiophosphates (Metcalf & March, 1953) and dimethylphosphoramides (Casida, Allen & Stahmann, 1953). However, attempts to demonstrate methylation in *Bombyx mori* and *Lucilia caesar* by the isolation of *N'*-methylnicotinamide following the feeding of nicotinamide have proved unsuccessful (Kato, 1953).

Aromatic and amino acids play an important role in the life of insects. Aromatic acids may serve as egg-hatching factors for mosquitoes (Abdel-Malek, 1948; Gjullin, Yates & Stage, 1939), and as insecticides or repellents (Bushland, 1940; Fennah, 1950; Swingle, Phillips & Gahan, 1944; and others). Although the toxicity of a few aromatic acids has been studied with mosquito larvae (Bodine, 1923; Bushland & King, 1943), no detailed studies on the structural relationship for aromatic acid toxicity have been reported. In regard to amino acids, isoleucine is required for oviposition by adult *Aedes aegypti* (Greenburg, 1951) and the larvae require ten amino acids for growth (Goldberg & DeMeillon, 1948). Paper chromatograms have identified twenty amino acids from adult Culicid mosquitoes (Clark & Ball, 1952) and eighteen from all the developmental stages of *Aedes aegypti* L. (Micks & Ellis, 1951, 1952).

The recent identification of a conjugation product such as hippuric acid (Friedler & Smith, 1953) in locusts does not necessarily indicate that the toxicity of benzoic acid to the insect was actually reduced by this conjugation. The ease of studying a detoxication problem with mosquito larvae bioassay prompted the use of this technique. The investigation reported here concerns the toxicity of various aromatic acids to mosquito larvae with particular reference to the counteracting influence of amino acids.

MATERIALS AND METHODS

Mosquito larvae. A small colony of *Aedes aegypti* L. was maintained (Casanges, McGovern & Chiles, 1949) as a source of eggs, which were collected on moist filter paper and dried so that they could be hatched when desired for use. The first-instar larvae generally used for assay were not fed before testing; later instars were fed on finely ground dog biscuits (Friskies Dog Food Meal) until the actual assay time, when only the experimental nutrients were provided. Larval instars were differentiated on the basis of head capsule width. The adult mosquitoes were fed 5% sucrose solutions except for a single blood meal immediately after emergence.

Compounds. The amino acids and aromatic acids tested were of the highest purity obtainable commercially. The samples of D- and L-glutamic acids were optically pure. The identities of the substituted benzoic acids were ascertained by melting points and neutralization equivalents. All amino acids were tested at 0·1 M concentration unless specifically stated otherwise. In the study of hydrogen-ion concentration, a buffer which was 0·02 M with respect to each of

acetate, orthophosphate and borate was used. All other studies utilized an 0·02M phosphate buffer, pH 7·0. The final pH of all solutions was confirmed before assay and did not change during the assay time.

Biological testing. Bioassays were made in 12 × 75 mm. tubes with 1·0 ml. total liquid volume per tube. The papers with the eggs were soaked in distilled water for about 8 hr., then the larvae were concentrated to about 20 larvae/drop by utilizing their negative phototropic response. To each assay tube was then added one drop (about 20 larvae) and the tubes kept at 27° for 24 hr., after which time the mortality was recorded. The mortality end-point was considered to be an inability of the larvae to move in response to a strong light source. Mortality comparisons were based on dosage/mortality curves with from 10–15 serial dilutions for each aromatic acid and a 1·3- to 1·6-fold dilution factor, depending on the precision desired for the results. The LD_{50} (molar concentration of toxicant effecting a 50% mortality) and confidence limits were determined by the method of Litchfield & Wilcoxon (1949) in which the probit of the percentage mortality was plotted against the logarithm of the molar concentration of toxicant. No consistent correlation of slope with relative toxicity or specific counteracting agents was evident.

The efficiency of an amino acid in these studies was considered to be its relative ability to decrease the toxicity of the aromatic acid involved. This was expressed as the 'toxicity index' which is the ratio between the LD_{50} of the aromatic acid alone and the LD_{50} in the presence of the amino acid, multiplied by 100 (Sun, 1950). Thus a figure of less than 100 would indicate that the amino acid decreased the toxicity of the aromatic acid.

Biosynthesis of hippuric acid. Three lots of about 300 fourth-instar *A. aegypti* larvae were starved for 24 hr. and then added to a solution containing 0·167M [α-^{14}C]glycine and 0·15M benzoic acid in 0·033M phosphate buffer at pH 7·0. After 36 hr. incubation at 25° the larvae were collected on filter paper, washed with distilled water and then homogenized (Potter & Elvehjem, 1936). The larval homogenate, the supernatant fluid in which the larvae had been swimming, and a sample of the glycine–benzoic acid solution comparable to that in which the larvae were originally suspended were then acidified with H_2SO_4 and extracted with a butanol–chloroform mixture according to the method of Chantrenne (1951). No [^{14}C]glycine was extracted into the organic phase, whereas a radioactive material formed by the mosquito larvae, presumably hippuric acid, appeared in this layer.

Further proof of the structure of the biosynthesized material extracted by the butanol–chloroform mixture was afforded by paper chromatography and co-chromatography with known samples of glycine and hippuric acid. For this purpose the solvent system used by Fewster & Hall (1951) for organic acids was modified to utilize the organic phase from a mixture of 60 ml. *n*-butanol, 30 ml. 95% (v/v) ethanol and 30 ml. of a 1·5N solution of ammonium carbonate and ammonia. Whatman no. 1 paper was used in upward-flow chromatography (Wolfson, Cohn & Devaney, 1949) at 25° and the glycine spot with a R_F of 0·11 was localized with ninhydrin while the hippuric acid of R_F 0·48 was detected with a pH indicator reagent of methyl red and bromophenol blue. The radioactive materials after incubation with mosquito larvae were co-chromatographed with known glycine and hippuric samples and the position and shape of the coloured spots compared with a radioautograph.

RESULTS

It was found in preliminary experiments that mosquito larvae were sensitive to aromatic acids and that this toxic action was reversed by gelatin and certain amino acids. Of twelve possible counteracting materials tested in combination with benzoic acid, all except L-cysteine and α-D-glucuronic acid decreased the toxicity of the benzoic to the mosquito larvae significantly.

Effect of larval instar. The toxicity of benzoic acid was tested against the four larval instars of *A. aegypti* (instars differentiated on the basis of head capsule width). The fourth instar was only about half as sensitive to the benzoic acid as were the three younger stages, which did not differ significantly in their susceptibility. The toxicity of benzoic acid to all instars was significantly reduced in the presence of either glycine or DL-ornithine (0·1M). The amino acids were the most efficient in reducing the toxicity of the benzoic acid with the first-instar and the least with the fourth-instar larvae, while the second and third instars did not differ significantly in this respect. Therefore, newly hatched first-instar larvae were selected as the best assay system for further study.

Influence of pH. A study of the effect of pH on the toxicity and counteraction by amino acids was made since both glycine and benzoic acid are partially ionized at pH 7·0. The toxicity of the benzoic acid increased significantly as the pH was lowered from 9·0 to 4·0. Compared with the toxicity at pH 7·0, benzoic was only two-thirds as toxic at pH 9·0, but was over 10 times as toxic at pH 5 and 20 times at pH 4·0. The counteracting influence of glycine was lost above pH 7·0, suggesting that the undissociated or cationic forms were primarily responsible for this reduction in toxicity. Further assays were standarized at pH 7·0.

Effect of amino acids. The effect of amino acids in counteracting the toxicity of benzoic acid is shown in detail in Table 1. The following relationships are evident: (1) Glycine and readily available sources of glycine such as glycylglycine and gelatin were the best counteracting agents tested. (2) The efficiency decreased with increasing chain length as shown with the glycine, alanine, valine, and leucine series, serine and threonine, and ornithine and lysine. The only discrepancy in this relationship was with glutamic acid or glutamine and aspartic acid or asparagine. (3) Serine and threonine were fairly comparable with their non-hydroxyl analogues. (4) The thio amino acids required the SH group blocked for counteraction, cf. cysteine with methionine and cystine. (5) Dicarboxylic acids were better than their amides. L-Glutamic acid was not significantly different from the D- or DL- acids. (6) The amino group is most efficient in the

Table 1. *Efficiency of various amino acids in counteraction of benzoic acid toxicity*

Two series of experiments designated as *A* and *B* are shown. In series *A* the LD_{50} for benzoic acid was 0·028 (0·025–0·031) M, while in series *B* the value was 0·023 (0·020–0·026) M. The toxicity index is the ratio between the molar LD_{50} value with benzoic acid alone and the molar LD_{50} of benzoic in the presence of 0·100 M amino acid, multiplied by 100. Each toxicity index is followed by the confidence limits for 19/20 probability. Those amino acids designated by * were tested partially in suspension, while others were in solution.

	Toxicity index
Series *A*	
Varying chain length	
Glycine	51 (45–55)
DL-Alanine	59 (55–65)
DL-Valine	56 (54–59)
DL-Isoleucine	62 (57–67)
L-Leucine	73 (68–78)
DL-Norleucine	73 (67–86)
Hydroxy amino acids	
L-Serine	57 (51–63)
DL-Threonine	65 (60–70)
Thio amino acids	
L-Cystine*	68 (62–74)
DL-Methionine	79 (74–85)
L-Cystine	120 (113–128)
Aromatic amino acids	
L-Proline	58 (54–62)
DL-Tryptophan	66 (62–70)
DL-Phenylalanine*	71 (66–76)
L-Tyrosine	79 (73–86)
L-Histidine	96 (89–102)
L-Hydroxyproline	105 (96–115)
Position of amino group	
DL-α-Alanine	59 (55–65)
β-Alanine	75 (68–82)
Availability of α-amino group	
Glycine	51 (45–55)
Glycylglycine	51 (48–55)
Betaine	118 (112–125)
Creatine	82 (74–90)
Creatinine	96 (87–106)
Series *B*	
Control	
Glycine	28 (26–30)
Isomers of glutamic acid	
L-Glutamic	42 (39–46)
D-Glutamic	45 (43–47)
DL-Glutamic	45 (41–50)
Dicarboxylic amino acids and their amides	
L-Glutamic	42 (39–46)
L-Glutamine	50 (45–56)
L-Aspartic	46 (42–51)
L-Asparagine	69 (63–77)
Basic amino acids	
DL-Arginine	39 (34–44)
DL-Ornithine	46 (42–50)
L-Lysine	51 (47–56)
DL-Citrulline	65 (58–73)

α-position (cf. α- and β-DL-alanines) and must be free as shown with betaine and glycine or glycylglycine, and with creatine and creatinine. (7) Of possible metabolic significance are the marked differences between arginine and citrulline, cystine and cysteine, and proline and hydroxyproline.

Effect of different levels of glycine, arginine and p-hydroxybenzoic acid on benzoic acid toxicity. Competition experiments were made with the two most efficient amino acids, glycine and arginine, and with the least toxic benzoic acid derivative, *p*-hydroxybenzoic acid. Fig. 1 shows this relationship.

With glycine the reduction in toxicity of benzoic acid was almost directly proportional to the logarithm of the glycine concentration up to 0·05 M, after which no further change resulted. At this optimum point in the curve, the benzoic acid and glycine were equimolar. At the three lowest concentrations the glycine appeared to reduce the toxicity of the benzoic acid as if a mole-for-mole detoxication were involved. Arginine was less effective, reached a lower optimum and was toxic itself at concentrations of 0·1 M. On the other hand, *p*-hydroxybenzoic acid increased the toxicity of benzoic acid up to 0·05 M in such a manner as to suggest a synergistic action since by itself it is non-toxic in this concentration range.

In vivo *hippuric acid synthesis by mosquito larvae.* A preliminary test demonstrated the presence of [^{14}C]hippuric acid in first-instar mosquito larvae incubated with [α-^{14}C]glycine and benzoic acid. When about 100 fourth-instar larvae/ml. were incubated for 36 hr. at 25° with 0·15 M benzoic acid

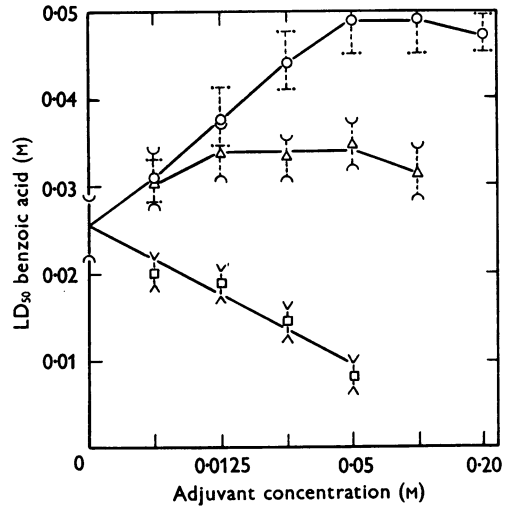

Fig. 1. Effects of glycine, arginine and *p*-hydroxybenzoic acid on the toxicity of benzoic acid. For conditions see the text. ○, glycine; △, arginine; □, *p*-hydroxybenzoic acid.

and 0·167 M [α-^{14}C]glycine, the larvae contained 12 % of the radioactivity, the remaining 88 % being in the supernatant. Of the latter radioactivity, 27·3 % was present as hippuric acid, whereas 43·5 % of the ^{14}C in the larvae was present as this acid. The identity of the biosynthesized radioactive hippuric acid was ascertained when co-chromatography with the authentic acid yielded a spot which was identical in position and shape to that found on the radioautograph of the biosynthesized material. In the radioautograph a very small amount of a faster moving component (R_F 0·70) also appeared.

These results indicate that mosquito larvae form hippuric acid *in vivo* in the presence of glycine and benzoic acid and that the conjugated derivative is probably excreted.

Relation of structure of aromatic acids to toxicity. Structural specificity studies for these aromatic acids are summarized in Table 2. The *para* substitution by halogens appeared to be the most effective and the *ortho* substitution the least in enhancing the toxicity of these aromatic acids. This was particularly evident with 2:6-dichlorobenzoic acid. Iodo derivatives were generally more toxic and displayed a different structural specificity than did the chloro derivatives. The most toxic substituted benzoic acid found was 2-hydroxy-3:5-diiodobenzoic acid, which was 5 times as toxic as 2:3:5-triiodobenzoic acid. The toxicity of 3:5-diiodobenzoic acid was increased by an *ortho*-hydroxyl or *para*-iodo group, but was decreased by an *ortho*-iodo substitution. This indicates that different combinations of ring substituents might yield still more toxic compounds. The *para*-chloro derivatives of benzoic, phenylacetic, diphenylacetic and phenoxyacetic acids were all more toxic than the parent acids. Of ten different *para* substituents, the iodo was the most toxic and the hydroxyl the least. An incomplete study indicates that the toxicity of the halogen generally increased and the glycine detoxifying efficiency decreased as the electronegative properties of the *para* halogen decreased. Side-chain variations greatly altered the activity in the phenyl series. Increasing the chain length in a normal aliphatic series decreased the toxicity. Phenoxy compounds were poorer than phenyl with either phenylacetic or diphenylacetic derivatives. Benzyl alcohol was 4 times as toxic as either benzoic acid or benzaldehyde. The sulphinic and phosphonic acid side chains were essentially inactive. Benzoic acid was 15 times as toxic as its glycine conjugate, hippuric acid. Among the ring variations, benzoic was more toxic than nicotinic or *cyclo*hexanecarboxylic acids. The acids containing five-membered heterocyclic rings were more toxic than benzoic acid, the thiophene and furane rings being comparable. A β-carboxyl was more effective than α- in both napthoic and thiophenic acids. Among the five diphenyl and diphenoxyacetic acids, the most toxic was di-*p*-chlorophenylacetic acid, an oxidation product and metabolite of the insecticide DDT (White & Sweeney, 1945).

Table 2. *Structural specificity for aromatic acid toxicity*

The figures are the molar LD_{50} values × 10^3, followed by the confidence limits for 19/20 probability.

para Substituents in benzoic acid

Iodo	4·9 (4·2–5·8)
Chloro	8·7 (7·7–9·8)
Bromo	9·3 (8·3–11)
Fluoro	14 (13–16)
Methyl	22 (19–25)
Hydrogen	23 (19–27)
Methoxy	25 (23–27)
Amino	29 (26–32)
Nitro	30 (27–34)
Hydroxyl	51 (45–58)

Halogenated benzoic acids

	Chloro	Iodo
2	24 (22–26)	—
3	10 (9·2–11)	—
4	8·7 (7·7–9·8)	4·9 (4·2–5·8)
2, 4	15 (13–17)	—
2, 5	17 (15–20)	25 (21–30)
2, 6	61 (55–67)	—
3, 4	5·3 (4·7–6·0)	3·7 (3·4–4·0)
3, 5	—	1·4 (1·3–1·5)
2, 3, 5	15 (13–17)	5·3 (4·5–6·1)
3, 4, 5	—	0·29 (0·27–0·30)
2, 3, 4, 5, 6	11 (10–12)	—

Ring variation

Benzoic	23 (19–27)
*Cyclo*hexanecarboxylic	63 (53–75)
Nicotinic	46 (40–54)
1-Naphthoic	35 (30–40)
2-Naphthoic	12 (10–14)
2-Furoic	11 (9·1–13)
2-Thiophenecarboxylic	13 (11–15)
3-Thiophenecarboxylic	9·4 (8·1–11)

Side chain variation: R = 

R.COOH	23 (19–27)
4-chloro	8·7 (7·7–9·8)
$R.CH_2.COOH$	26 (23–30)
4-chloro	9·7 (8·4–11)
$R.CH_2.CH_2.COOH$	37 (32–43)
R.CH=CH.COOH	29 (28–31)
$RO.CH_2.COOH$	69 (66–72)
4-chloro	46 (42–50)
$RO.CH_2.CH_2.COOH$	42 (37–48)
RCHO	25 (24–26)
RCH_2OH	5·5 (5·2–5·8)
$R.CO.NH.CH_2.COOH$	340 (300–390)
RSO_2H	440 (410–480)
RPO_3H_2 (3-chloro)	280 (260–300)

Miscellaneous aromatic acids

Diphenylacetic	47 (41–54)
Di(*p*-chlorophenyl)acetic	0·20 (0·19–0·22)
Di(*p*-methoxyphenyl)acetic	41 (33–52)
Di(*p*-chlorophenoxy)acetic	14 (10–21)
Di(*o*-chlorophenoxy)acetic	33 (27–39)
Indole-3-acetic	52 (49–55)
2-Furanacrylic	45 (39–52)

DISCUSSION

Studies of structural specificity of amino acids for the detoxication of aromatic acids have been limited previously by the lack of a suitable assay system. It is known that detoxication reactions involving glycine, ornithine and glutamine may occur in different animals (Williams, 1949). The results from this study with mosquito bioassay should be of particular interest, since they greatly expand the amino acids known to be effective in reducing the toxicity of aromatic acids. It is not known which ones act directly and which require a preliminary metabolic conversion into the effective counteracting agent. The structural specificity of this interaction was previously discussed, but several points seem worthy of further consideration. Asparagine and hydroxyproline, two of the poorer amino acids in counteraction, are not found in mosquitoes (Clark & Ball, 1952; Micks & Ellis, 1951, 1952), whereas the more efficient glutamine and proline are. Also, proline is metabolized in certain organisms to yield glutamic acid (which was shown to be very good in counteraction), whereas hydroxyproline is not (Umbreit, 1952). Decreases in efficiency with chain length are possibly due to penetration difficulties. Other still unexplained points of interest are: the poor relative efficiency of the thio amino acids compared with the hydroxy amino acids; the better efficiency of glutamic than of aspartic acid; the better efficiency of dicarboxylic acids than of their amides; and the better action of arginine than of citrulline.

The activities of benzoic acid and its derivatives have been studied with many biological systems, but in no case where adequate studies have been made for comparison is the relation of structure to toxicity the same as for the mosquito larvae. Structural specificity for the inhibition of the most sensitive enzyme reported, D-amino acid oxidase (Bartlett, 1948), varies greatly from the specificity for this insect bioassay. However, this does not rule out the possibility that the same physiological mechanism is involved in the toxicity, since the barrier of the insect cuticle may account for certain of the specificity differences. Many insecticides display their greatest toxicity in the non-ionized form (Brown, 1951), as was the case with benzoic acid in this study, due to the inability of ionized molecules to penetrate through the insect cuticle readily.

The interaction of different benzoic acid and amino acid levels illustrates a synergistic action of insecticides and other toxicants. Glycine, arginine and many other amino acids naturally present in insects were shown to reduce the toxicity of benzoic acid. At non-toxic concentrations, *p*-hydroxybenzoic acid increased the toxicity of benzoic acid. Both could presumably be involved in a glycine conjugation reaction. The enzymic conjugation forming *p*-aminohippuric acid may be inhibited by structurally related compounds including hydroxybenzoic acids (McKinney, 1951). It would appear that *p*-hydroxybenzoic acid might have effected its synergistic action by competition for the detoxication sites with benzoic acid, and in so doing reduced the amount of benzoic acid lost to detoxication and thereby increased its toxicity. This would be an example of synergistic action through competition for a common detoxication mechanism.

SUMMARY

1. The toxicity of benzoic acid to *Aedes aegypti* L. larvae was reduced in the presence of glycine. The efficiency of this counteraction decreased as the larvae matured and was greatest at pH 7·0 or below. The quantitative relationship of the decrease in benzoic acid toxicity in the presence of low glycine concentrations suggested an equimolar detoxication reaction. Benzoic acid was 15 times as toxic as hippuric acid to these larvae. Using [α-^{14}C]glycine it was shown that mosquito larvae can synthesize hippuric acid and probably excrete the conjugated product.

2. Thirty amino acids and closely related compounds were tested for their efficiency in reducing the toxicity of benzoic acid. Although glycine was the most efficient amino acid, in its absence many others were capable of reducing the toxicity of benzoic acid. The structural specificity for this counteraction is discussed.

3. The toxicity of fifty different aromatic acids or closely related compounds was determined. The relation of structure to the toxicity and mechanism of action of these acids is discussed.

4. An example is presented for a synergistic action through competition for a common detoxication mechanism.

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Canavanine: Detection and Occurrence in *Colutea arborescens*

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Canavanine, α-amino-δ-guanidinoxybutyric acid, is the only known example of a natural compound containing the guanidinoxy function,

$$—O.NH.C(:NH).NH_2.$$

Although canavanine occurs free to the extent of 2–3·5 % of dry weight in the seeds of the jack bean, *Canavalia ensiformis*, and the closely-related *C. lineata*, its discoverer, M. Kitagawa, was unable to obtain it from any other leguminous plant (Kitagawa & Tomiyama, 1929; Kitagawa, 1937). It has, however, been found in the seeds of *C. obtusifolia* (Damodaran & Narayanan, 1939).

During a study of the colour reactions of pentacyanoferrate derivatives and substituted guanidines, it was observed that guanidinoxy compounds, including canavanine, reacted only within the range pH 5–7·5, whereas the alkyl-substituted guanidines, including arginine, reacted only in solutions more alkaline than pH 8 (Fearon, 1946). This is in agreement with the observations of Kitagawa (1937) and Archibald (1946). As the result of an application of the pentacyanoferrate reactions to a survey of plant tissues, it has now been possible to show the presence of a guanidinoxy compound in some species of *Medicago* and *Ornithopus*, and canavanine has been isolated from the seeds of the bladder senna, *Colutea arborescens*.

EXPERIMENTAL

Reagents

A freshly prepared neutral aqueous solution of disodium pentacyanonitrosoferrate (sodium nitroprusside) does not react chromatically with a guanidoxine until the mixture has been exposed to daylight for about 5 min., when a colour, ranging from orange-red (*N*-methoxyguanidine) to magenta (canavanine) develops, and may persist for days. The pigment is stable to dilute acid, but is reversibly bleached by alkali. Alternatively, an aqueous solution of nitroprusside may be activated by irradiation before doing the test (Kitagawa & Yamada, 1932), though this seems to