The Estimation of Aromatic Amidines

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The investigation of a series of aromatic amidines possessing anti-typhus (Andrewes, van den Ende, King & Walker, 1944) and anti-bacterial (Evans, Fuller & Walker, 1944) actions made necessary a quantitative method for their estimation. When aromatic amidines are heated in alkaline solution with glyoxal, a complex series of coloured compounds is formed (Ekeley & Ronzio, 1935, 1937). A study of the effect of pH and the amount of glyoxal showed that heating the reactants at pH 12 gave a purple colour, but this was not sensitive enough for clinical use. While using different buffers it was found that sodium borate is a catalyst for the reaction, and that in its presence at pH 9 a brilliant stable magenta colour was produced, in dilutions down to 1 in 100,000 of amidine. When the method had been in use for some time, a paper appeared describing a process of estimation also using glyoxal (Devine, 1944). This uses the brown colour produced when an amidine is heated with a large excess of glyoxal (about 2000 times the amount used in the present method) in 0.6N-NaOH. It has the disadvantages that the glyoxal reagent is unstable and must be made up daily; that the conditions of heating (30 sec. in the boiling water-bath) are difficult to reproduce exactly; and that the colour is unstable and must be estimated immediately. It is, however, not so sensitive to variations in the amount of glyoxal used as the method to be described.

METHODS

Reagents

Glyoxal reagent. 0.1% aqueous solution of glyoxal sodium bisulphite (British Drug Houses, Ltd.). This is stable for several weeks if kept in an amber bottle.

Borate buffer. 4 g. of boric acid are neutralized to pH 9 (neutral to thymol blue, faintly pink to phenol phthalein), made up to 100 ml. and heated to dissolve.

Procedure

10 ml. of solution, containing 1–4 mg./100 ml. of amidine, are heated in a boiling water-bath for 10 min. with 1 ml. of glyoxal reagent and 1 ml. of borate buffer. After cooling and acidifying with 0.2 ml. of 2N-HCl, the colour is examined either in an absorptiometer using an Ilford Spectrum Green filter, or against standards in a visual colorimeter. With many amidines, the colour becomes pinker in acid, and bluer in alkaline solution, and although the acidification somewhat reduces the intensity of the colour, it is advisable since many biological fluids are cloudy at alkaline reactions.

The colour is maximal at 1–2 mol. of glyoxal/molecule of amidine, and excess of glyoxal inhibits colour production. This is shown in Fig. 1, which gives the colour production ('Spekker' absorptiometer readings) by V187 (*p*-methylsulphonylbenzamidine) with different amounts of glyoxal.

Fig. 1. Effect of strength of glyoxal reagent on the colour production by V187 (*p*-methylsulphonylbenzamidine). Curve 1, 1 mg./100 ml.; curve 2, 2 mg./100 ml.; curve 3, 3.5 mg./100 ml.; curve 4, 5 mg./100 ml.; curve 5, 7.5 mg./100 ml.; curve 6, 10 mg./100 ml. of V187.

Fig. 2 gives the 'Spekker' absorptiometer curves for fixed glyoxal and increasing amidine concentrations. These show a preliminary curved part, where excess of glyoxal depresses colour production, and a flattening out where the glyoxal is insufficient to combine with all the amidine. With the strongest glyoxal reagent (0.5%), the preliminary curve is accentuated, while with the weakest (0.05%) the curve soon flattens out. Only over a portion of the curve is the colour intensity proportional to the strength of the amidine. It follows that any one strength of glyoxal is suitable for only a limited range of amidine concentration. 0.1% glyoxal was chosen as it gives maximum sensitivity with the lowest concentrations of amidine. It must be remembered that (1) if an intense reaction is obtained,

the test must be repeated after dilution of the amidine in case the amount of glyoxal is insufficient for complete colour production, and (2) for accurate results the concentrations of standards and unknowns must not be very different, particularly if these are low.

Fig. 2. Spekker absorptiometer curves for V187 using different strengths of glyoxal reagent. Curve 1, 0.05%; curve 2, 0.1%; curve 3, 0.2%; curve 4, 0.35%; curve 5, 0.5% of glyoxal sodium bisulphite.

Specificity of the reaction

The reaction is specific for an unsubstituted amidine directly joined to an aromatic nucleus. Nearly fifty aromatic amidines, including ring-substituted benzamidines, naphthalene diamidine and nicotinamidines, have been tested, and all give the reaction. It is not given by an aromatic amidine with one or two methyl groups on the N atoms of the amidine group, or by benzamidrazones or phenyl acetamidines. Guanidines, biguanides, or aliphatic amidines do not react.

Estimation of V187 in blood

As an example of the estimation of an amidine, that of *p*-methylsulphonylbenzamidines (V187) is described. This drug is slowly absorbed from the gut, and is quickly excreted (Evans *et al.* 1944), and blood amidine concentrations will be very low except in experimental conditions. This rapidity of excretion is partly explained by the presence of most of the drug in the plasma. When 20 mg./100 ml. were added to horse blood, 7 mg./100 ml. were present in the red cells and 34 mg./100 ml. in the plasma.

(a) For concentrations over 1 mg./100 ml. Amidines are precipitated by many protein precipitants, and are also absorbed by filter paper. Tungstic acid, trichloroacetic acid, toluenesulphonic acid, metaphosphoric acid, and zinc hydroxide were all unsatisfactory as protein precipitants. Precipitation with methanol allowed some amidine to pass into the filtrate; ethanol was better, and isopropanol gave satisfactory recovery.

1 ml. of blood and 4 ml. of isopropanol are well mixed and filtered by gravity or suction. 2 ml. of filtrate are placed in a 6 in. $\times$ $\frac{5}{8}$ in. tube, graduated at 2.5 ml., with 0.2 ml. of borate, 0.2 ml. of glyoxal reagent and a fragment of porous pot. The mixture is heated in a boiling water-bath for 10 min. Standards containing 0.5, 1.0 and 2.0 mg. of amidine/100 ml. are similarly treated. The contents of the tubes need heating carefully at first to avoid frothing. (This risk may be minimized by using tubes consisting of a 1.5 in. $\times$ 0.5 in. tube fused to a 6 in. length of 1 in. tubing.) When cool, the mixtures are acidified with a drop of 2N-HCl and diluted to the mark with isopropanol. The acid and alcohol are necessary to dissolve the borates and fat. The alcohol is boiled off during the test to make the reaction more sensitive by increasing the boiling-point of the mixture and increasing the concentration of the reactants.

(b) For concentrations, under 1 mg./100 ml. (if the pigment is fluorescent). The pigments from V187 and from some other amidines are fluorescent in ultra-violet light in acid solution down to 1 μ g./100 ml. *iso*Propanolic extracts of blood cannot be used because they give a fluorescent blank, but dialyzed iron may be used for deproteinizing in a method similar to that of Devine (1944). The correct strength of dialyzed iron necessary to precipitate the blood proteins without leaving excess must be found. In these tests, dialyzed iron (British Drug Houses, Ltd.) diluted to 70% was found satisfactory.

1 ml. of blood, 3 ml. of water and 1 ml. of dialyzed iron are mixed and heated in a boiling water-bath with stirring until the mixture coagulates to a brownish mass (1 min.). It is spun down and filtered clear through a sintered glass filter. 2 ml. of filtrate, 0.2 ml. of borate and 0.2 ml. of glyoxal reagent are heated in the boiling water-bath for 10 min., along with standards made from 0.2, 0.02 and 0.002 mg./100 ml. Standards and unknowns are compared in a fluorimeter after making acid or alkaline according to the conditions under which the pigment is most fluorescent, and the results read off the standard curves.

Estimation in urine

The urine must be neutralized to thymol blue or phenolphthalein. If the content of amidine is unknown, it is tested undiluted and at 10- and 100-fold dilutions.

10 ml. of urine, 2.5 ml. of borate, and 1 ml. of glyoxal reagent are heated in the boiling water-bath for 10 min., cooled and acidified with 1 ml. of 2N-HCl. The suitable dilution is compared with a standard, and the dilution

necessary to give 2-3 mg./100 ml. is judged, and the test repeated with two dilutions either side of this.

As with most drugs, low concentrations of amidines cannot be estimated quantitatively in urine owing to the interfering substances present. On the average, 30 mg./100 ml. gave 65 %, 50 mg./100 ml. gave 75 % and 100 mg./100 ml. gave 85 % recovery.

The possibility of the excretion of conjugated amidines was not investigated.

Estimation in faeces

The sample is rubbed up with sufficient water and dilute acid (5 vol. or more) to give an easily flowing mixture acid to congo red, and is centrifuged. The extract is neutralized, mixed with 4 vol. of isopropanol and filtered. The estimation is carried out as for blood, but further dilutions may be necessary. This procedure is necessary in order to keep back chlorophyll and other isopropanol-soluble pigments.

Examples of the practicability of the method are described in the paper by Evans *et al.* (1944).

Estimation of propamidine

The concentrations of this drug which can be tolerated in blood are too low to be estimated colorimetrically by this or Devine's method, and since the glyoxal pigment is not fluorescent, the question of estimation in blood does not arise. The conditions for getting maximal colour are similar to those for V187, but the pigment is sparingly soluble in water, although soluble in dilute acetic acid. The method for urine is therefore slightly altered.

10 ml. of urine (and dilutions thereof), 2.5 ml. of borate and 1 ml. of glyoxal reagent are heated for 10 min. in a boiling water-bath, and while still hot, acidified with 2 ml. of glacial acetic acid. The pigment is estimated by comparison with standards, or on the Spekker absorptiometer.

Estimation of stilbamidine

Devine describes the estimation of stilbamidine, but does not give examples of analyses of actual blood samples. It is doubtful whether his method, or the present colorimetric method, is sensitive enough to detect the blood concentrations met with in practice.

Stilbamidine itself is fluorescent, down to 0.1 mg./100 ml., and a simple fluorimetric estimation on a dialyzed iron filtrate would therefore suffice for estimations down to 0.5 mg./100 ml. in blood. The pink glyoxal pigment is fluorescent in acid solution down to 0.01 mg./100 ml., and the general fluorimetric method will estimate stilbamidine in blood down to 0.05 mg./100 ml.

In urine or other aqueous solution, the colorimetric estimation is satisfactory. The glyoxal pigment is very sparingly soluble, even in dilute acetic acid, and so, after acidifying, an equal volume of ethanol is added to keep it in solution.

RESULTS

Table 1 gives the blood concentrations and Table 2 the rates of excretion of *p*-methylsulphonylbenzamidine (V187) by guinea-pigs after receiving 100 mg. of the drug by the intramuscular or oral route. The absorption and excretion of the amidine are rapid after an intramuscular dose, and slow and incomplete after an oral dose. The blood estimations on animals 4 and 5 were made by the fluorimetric method, and all the other tests by the colorimetric method.

SUMMARY

The use of glyoxal in the estimation of aromatic amidines is described.

I wish to thank Dr H. King and Dr J. Walker for supplying the compounds, and Dr D. G. Evans for the animal experiments.

Table 1. *Blood amidine concentration of guinea-pigs after 100 mg. of V187*

Animal no.	Route	Blood concentration (mg./100 ml.) at hr. after dose									
		$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{4}$	1	2	5	6	24	48	72
1	Intramuscular	4.7	4.7	3.2	3.0	—	<1.0	—	—	—	—
2	"	4.8	5.3	4.9	3.2	—	<1.0	—	—	—	—
3	"	3.3	3.3	3.6	3.0	—	1.0	—	—	—	—
4	By mouth	—	—	—	—	0.2	—	0.25	1.1	0.3	0.04
5	"	—	—	—	—	0.4	—	0.2	1.0	0.1	0.06

Table 2. *Excretion of amidine by guinea-pigs after 100 mg. of V187*

Animal no.	Route	Drug in urine (mg.) hr. after dose							Drug in faeces (mg.) hr. after dose			Drug in intestine at 72 hr. (mg.)	Total in faeces (mg.)	Recovery (mg.)
		0-3	3-6	6-24	0-24	24-48	48-72	0-72	0-24	24-48	48-72			
1	Intramuscular	27.6	30.7	19.9	78.2	—	—	—	—	—	—	—	—	78.2
2	"	52.0	19.4	7.0	78.4	—	—	—	—	—	—	—	—	78.4
3	"	24.2	38.3	16.2	78.7	—	—	—	—	—	—	—	—	78.7
4	By mouth	—	—	—	4.4	4.5	2.9	11.8	15.4	11.0	3.7	7.2	37.3	49.1
5	"	—	—	—	10.4	3.1	1.2	14.7	11.0	5.2	3.0	13.8	33.0	47.7

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The Amylase and Maltase of *Clostridium acetobutylicum*

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This work concerns the degradation of starch to glucose by enzymes of *Clostridium acetobutylicum*, a stage in the butanol-acetone fermentation which has so far been little investigated. Previous workers have mainly used growing cultures of *Cl. acetobutylicum*, though Robinson (1922) showed that starch (and other carbohydrates) were fermented by toluene-treated cell suspensions. In the present work we have used cell-free enzyme preparations obtained from culture filtrates of *Cl. acetobutylicum* (Weizmann). If the organism be grown in media containing maltose as the only carbohydrate, the culture filtrates contain a maltase but have no starch-splitting activity; if grown in media containing starch, culture filtrates contain both a maltase and an amylase. The properties of both these enzymes have been studied, and the amylase has been partially purified.

METHODS

Estimation of reducing sugars. The activities of both enzymes were followed by measurement of the reducing groups set free by their action, using a modification (C. S. Hanes, unpublished) of the method of Somogyi (1937).

Solution A is made by dissolving 50 g. anhyd. Na_2CO_3 , 25 g. Rochelle salt, 7.5 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 160 g. anhyd. Na_2SO_4 and 1.5 g. KI, in the order given, in 750 ml. boiling distilled water; on cooling, 0.75 g. KIO_3 is added and the volume made up to 1 l. It is stored at 37°.

Solution B is a mixture of equal volumes of 5% KI and 5% neutral potassium oxalate, mixed immediately before use.

The unknown solution (5 ml.), containing up to 3 mg. maltose or 1.5 mg. glucose, is heated with 5.0 ml. of solution A in a boiling water-bath for 15 min. After rapid cooling to room temperature, 2 ml. solution B, followed by 3 ml. 2N- H_2SO_4 , are added; the solution is allowed to stand 3 min., and titrated with 0.01M- $\text{Na}_2\text{S}_2\text{O}_3$, with 1% starch in saturated NaCl as indicator.

The calibration figures for maltose and glucose are:

Titre (ml. of 0.01M- $\text{Na}_2\text{S}_2\text{O}_3$) with	Amount of sugar taken (mg.)						
	0.5	1.0	1.5	2.0	2.5	3.0	3.5
(a) for maltose	0.92	1.92	2.91	3.90	4.91	5.97	7.05
(b) for glucose	1.78	3.62	5.53	7.47	—	—	—

Measurement of amylase activity

Reduction method. We define 1 reduction unit of amylase activity as that amount of enzyme which produces reducing substances equivalent to 7.5 mg. maltose in 30 min. at 37°, when allowed to act on 2 ml. 1% soluble starch and 1 ml. M-acetate buffer of pH 4.6, in a total volume of 4 ml. Mixtures were deproteinized by the method of Somogyi (1931) and reducing sugar in a portion of the filtrate determined by the above technique. Since the amount of reducing sugar produced is not a linear function of the enzyme concentration (Fig. 4), the number of reduction units of enzyme in an unknown solution is found by interpolation from this standard curve.

Iodine colour method. *Cl. acetobutylicum* amylase brings about the conversion of starch to substances giving no colour with iodine. Suitably diluted enzyme solution (2 ml.) is mixed with 1 ml. 1% soluble starch and 1 ml. M-acetate buffer (pH 4.6) and incubated at 37°. Samples are withdrawn at intervals and tested with iodine, and the time taken to reach the 'achromic point' noted. The iodine-colour unit of amylase activity is defined as the amount of enzyme which reaches the achromic point in 1 hr. under the above conditions. Up to an enzyme concentration of 20 units, the time taken to reach the achromic point is inversely proportional to the enzyme concentration.

The iodine colour method is more convenient than the reduction method, and though less accurate, is useful for preliminary experiments. When the same enzyme solution is measured by both methods, it is found that 1 reduction unit = 7.1 iodine-colour units.

Measurement of maltase activity

We define one maltase unit as the amount of enzyme bringing about the hydrolysis of 5 mg. maltose in 1 hr. at 37°, when allowed to act on 2 ml. 1.5% maltose and 1 ml. 0.2M-acetate buffer of pH 4.25, in a total volume of 6 ml. The methods used for deproteinization and measurement of reducing substances were as previously described. The relationship between enzyme concentration and maltose hydrolyzed is linear over the range 0.5–1.5 maltase units.

RESULTS

The maltase of *Cl. acetobutylicum*

Preparation of the maltase. Culture filtrates of *Cl. acetobutylicum* grown in a maize-meal medium