Absorption and Excretion of 4-Hydroxyisophthalic Acid

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(Received 5 October 1955)

In a preliminary communication, Chesher *et al.* (1955) reported that 4-hydroxyisophthalic acid, which had been shown to be the major constituent of the 'brown dust' left after purification of salicylic acid by sublimation, possessed analgesic activity when administered intraperitoneally to rats. Under these conditions it was more effective and less toxic than acetylsalicylic acid. Its antipyretic activity was of the same order as that of acetylsalicylic acid when tested on rabbits injected with a pyrogen solution prepared from *Proteus vulgaris*. The excretion of 4-hydroxyisophthalic acid was investigated to determine the fate of this substance in the body. The material used in the tests was obtained by extraction of 'brown dust' as described by Hunt, Jones & Lindsey (1955) and had m.p. 314–315° (decomp.).

METHOD AND RESULTS

Separation of 4-hydroxyisophthalic acid from related acids

In common with most other hydroxylated aromatic acids, 4-hydroxyisophthalic acid gives a colour with FeCl_3 solution; this has a more reddish tinge than the colours given by most of the other acids. It can also be distinguished from most of these acids by its behaviour towards other

reagents, and Table 1 summarizes the results obtained when such reagents were sprayed on strips of filter paper 'spotted' with solutions containing about 5 μg . of aromatic acids.

4-Hydroxyisophthalic acid can be separated from closely related acids by paper electrophoresis. The apparatus used was that described by Flynn & de Mayo (1951), a p.d. of 400 v being applied to the electrodes with 2N acetic acid as the electrolyte. After 16 hr., the strips of filter paper (Whatman no. 3) were removed from the apparatus, dried and sprayed with one of the reagents described in Table 1. The results indicate that 4-hydroxyisophthalic acid migrates in the direction of the anode and moves further from the point of application than any of the other acids examined, with the exception of 2-hydroxyisophthalic acid. It can readily be separated from both salicylic and gentisic acids by this method.

Detection of 4-hydroxyisophthalic acid and metabolites in urine

Paper electrophoresis was used to detect the presence of 4-hydroxyisophthalic acid and possible metabolites in human and rats' urine. In each case 50 μl . of urine was applied. Human urine was used undiluted, and rats' urine, collected over 24 hr., was diluted to a volume representing 10–15 ml. per rat. Normal human urine gave no colour with FeCl_3 on either side of the point of application after paper electrophoresis, whereas a 24-hr. sample of urine collected from subjects who had taken 0.66 g. of 4-hydroxyisophthalic acid by mouth gave a claret-coloured spot at a

Table 1. *Colour reactions on filter paper of various aromatic acids and phenol (about 5 μg .) and their behaviour on paper electrophoresis*

The reagents used had the following composition: (a) FeCl_3 solution (0.40% in acetone). (b) Ammoniacal AgNO_3 solution (Bray, Thorpe & White, 1950): 0.1N- AgNO_3 (25 ml.) and NH_4OH (8 ml. of sp.gr. 0.88) made up to 50 ml. with water. (c) Folin-Ciocalteu reagent: (i) Folin-Ciocalteu reagent, followed by (ii) a saturated solution of Na_2CO_3 . (d) Aniline/xylose (Browning reaction; Smith & Spriesterbach, 1954): 5% aniline and 5% D-xylose in 50% ethanol. After spraying, the strip is heated for a few minutes at 125–130°.

Compound examined	Test applied				Distance travelled (cm.)	
	(a) FeCl_3	(b) AgNO_3	(c) Folin-Ciocalteu	(d) Browning reaction	To anode	To cathode
2-Hydroxyisophthalic acid	Orange	No colour	No colour	Brown	16.0	—
4-Hydroxyisophthalic acid	Claret	No colour	No colour	Brown	6.7	—
5:6-Dihydroxyisophthalic acid	Violet	Grey	Dark blue	Brown	5.7	—
Salicylic acid	Mauve	No colour	Greenish blue	Brown	2.7	—
Gentisic acid	Transient blue	Grey	Blue	Brown	2.5	—
2:4-Dihydroxybenzoic acid	Reddish purple	No colour	Dark blue	Brown	0.5	—
4-Methoxyisophthalic acid	No colour	No colour	No colour	Brown	—	0.5
isoPhthalic acid	No colour	No colour	No colour	Brown	—	0.5
Protocatechuic acid	Greenish grey	Grey	Dark blue	Brown	—	0.7
p-Hydroxybenzoic acid	Pale yellow	No colour	Greenish blue	Brown	—	1.0
Phenol	No colour	No colour	Dark blue	No colour	—	1.0

point 6.5 cm. towards the anode from the point of application, corresponding closely with the position taken up by 4-hydroxyisophthalic acid. No coloured spots formed at the positions that would have been occupied by salicylic acid, gentisic acid, 2:4-dihydroxybenzoic acid, 5:6-dihydroxyisophthalic acid or protocatechuic acid, had these been present, and it is concluded that these acids are not metabolites of 4-hydroxyisophthalic acid. The presence of 4-methoxyisophthalic acid, *p*-hydroxybenzoic acid, isophthalic acid and phenol, however, could not be excluded, because normal human urine gave with the aniline-xylose reagent a spot at a position 0.2-0 cm. towards the cathode from the point of application, and this spot would obviously have obscured the presence of any of these substances.

Estimation of 4-hydroxyisophthalic acid in urine, faeces, blood and the alimentary tract

The formation of a claret colour with Fe^{3+} ions was made the basis of a method for estimating 4-hydroxyisophthalic acid in urine, faeces, blood and the alimentary tract, the method of Trinder (1954) for salicylic acid being used. The reagent consists of 40 g. of A.R. HgCl_2 , 40 g. of $\text{Fe}(\text{NO}_3)_3$ and 120 ml. of N-HCl made up to 1 l. with distilled water. For the estimation of 4-hydroxyisophthalic acid in urine the HgCl_2 was omitted.

A standard curve was prepared by plotting the concentrations against the optical densities of the claret-coloured solutions obtained by treating 1 ml. of aqueous solution containing 0.1-0.5 mg. of 4-hydroxyisophthalic acid with 5 ml. of reagent. The colour of these solutions was stable for at least 24 hr. Measurements of optical density were made in a Gallenkamp colorimeter, with an Ilford filter no. 623 and a control solution consisting of 1 ml. of water and 5 ml. of reagent. A straight line passing through the origin was obtained. A similar curve was obtained for the Ca salt at equimolar concentrations, and no variation was caused by carrying out the assay at pH 4, 7 or 9.

The aqueous fluid or extract to be assayed, diluted to contain 0.1-0.5 mg. of 4-hydroxyisophthalic acid per ml., was treated in the same way as a standard solution. To correct for interfering colours, the claret colour was discharged by the addition of 0.1 ml. of syrupy H_3PO_4 , and the residual colour was evaluated against water, the result thus obtained being subtracted from the original reading. The amount of 4-hydroxyisophthalic acid present was then determined from the standard curve.

Since urine from untreated rats gave with the reagent a colorimeter reading equivalent to the excretion of about 0.3 mg. of 4-hydroxyisophthalic acid per rat in 24 hr., a blank value was determined before administration of the drug. Similarly, normal human urine gave a colour equivalent to about 40 mg. excreted in 24 hr. The figures vary somewhat from day to day.

For the estimation of 4-hydroxyisophthalic acid in rat faeces, the dried material was ground to a powder, moistened with about 0.75 ml. of N-HCl per g. and extracted with ether in a Soxhlet apparatus for 7 hr. About 10 ml. of water was added to the extract and the ether distilled off. The aqueous extract was then neutralized with N-NaOH and made up with distilled water to contain 0.1-0.5 mg. of 4-hydroxyisophthalic acid per ml.

For the estimation of 4-hydroxyisophthalic acid in blood, a 1 ml. sample was withdrawn from each animal by heart puncture and added to 0.5 ml. of an anticoagulant solution

consisting of 0.4% of potassium oxalate and 0.6% of ammonium oxalate. A portion (1 ml.) of the mixture was immediately transferred to a centrifuge tube, treated with 5 ml. of the reagent and centrifuged. The optical density of the supernatant liquid was measured as described above.

For the estimation of 4-hydroxyisophthalic acid in the alimentary tract, extracts were prepared by grinding (a) the oesophagus and stomach, (b) the small intestine, and (c) the caecum and large intestine with sand and water. The suspensions were adjusted to pH 8-9 with 10% NaOH solution and were then boiled before being made up to a suitable volume for assay.

Recovery of 4-hydroxyisophthalic acid added to rats' urine

Amounts of 4-hydroxyisophthalic acid ranging from 0.1 to 0.5 mg. were added to each ml. of urine and the 4-hydroxyisophthalic acid content was estimated. The results from experiments carried out in triplicate indicated that the average recovery is $100.9 \pm 6.1\%$. When the values for 0.1 mg./ml. are omitted, the recovery is $100.1 \pm 3.8\%$.

Recovery of 4-hydroxyisophthalic acid added to rats' faeces

4-Hydroxyisophthalic acid (20 mg.) was added to the faeces excreted in 24 hr. by two rats. After extraction, 19.8 mg. of 4-hydroxyisophthalic acid was found on assay. This represents a recovery of 99%.

Recovery of 4-hydroxyisophthalic acid added to rats' blood

4-Hydroxyisophthalic acid (0.5 mg.) was added to 0.5 ml. of blood and the sample was assayed before and after incubation at 37° for 1 hr. Recoveries of 0.525 and 0.495 mg. were obtained, corresponding to percentage recoveries of 105 and 99%. When 0.2 mg. of 4-hydroxyisophthalic acid was added to 1.0 ml. of blood a recovery of 0.19 mg. was obtained, equivalent to 95% of the theoretical value.

Estimation of 4-hydroxyisophthalic acid in urine and faeces

4-Hydroxyisophthalic acid was administered, mixed with the food, to a pair of rats, 19 mg. being given to the two animals, and the amount present in the urine was assayed by the method described above. A recovery of 54% was obtained in the urine excreted during the first 24 hr. with no additional amount in the next 24 hr. sample. Rats were also each given by stomach tube doses of 10 and 50 mg. of 4-hydroxyisophthalic acid, a dose of 10 mg. of the acid with 100 mg. of NaHCO_3 and a dose of 18 mg. of the calcium salt of the acid (a hexahydrate), two rats being used in each experiment. The results are summarized in Table 2, and indicate that between 43 and 63% of the acid may be recovered in the urine and faeces within 24 hr. Not more than 4% was found in the faeces or in the urine within the next 24 hr.

In an attempt to determine the fate of the rest of the 4-hydroxyisophthalic acid, experiments were

Table 2. *Percentage of 4-hydroxyisophthalic acid excreted by pairs of rats after oral administration*

	Substance and amount administered per rat				
	Acid (50 mg.)	Acid (10 mg.)	Acid (10 mg.)	Ca salt (18 mg.)	Acid (10 mg.) + NaHCO ₃ (100 mg.)
First 24 hr. urine	20.8	37.8	35.0	28.1	33.8
First 24 hr. faeces	22.9	26.0	26.3	28.6	19.0
Total	43.7	63.8	61.3	56.7	52.8

Fig. 1. Rate of urinary excretion of 4-hydroxyisophthalic acid in rats. Oral and intraperitoneal administration of 20 mg./animal in groups of five as either the free acid or its sodium salt. $\circ$ — $\circ$, Acid, oral (average from two experiments); $\times$ — $\times$, sodium salt, oral (average from two experiments); $\triangle$ — $\triangle$, acid, intraperitoneal (average from two experiments); $\times$ — $\times$, sodium salt, intraperitoneal (average from three experiments).

carried out to determine whether some of the acid was being excreted in bound form. Samples of urine were hydrolysed with 2N-H₂SO₄, 3N-HCl, N-NaOH, a solution of KOH in ethylene glycol, and HI in a mixture of acetic anhydride and glacial acetic acid. In no instance, however, could the presence of a hydrolysable complex be demonstrated by an increase in the amount of colour produced.

Rate of urinary excretion of 4-hydroxyisophthalic acid

4-Hydroxyisophthalic acid was administered to two groups of five rats, each group being given 100 mg. by stomach tube and by the intraperitoneal route respectively, and samples of urine were collected at intervals during the next 24 hr. and assayed as before. Other groups of rats were given 124 mg. of the sodium salt (equivalent to 100 mg. of the free acid) by both routes and samples of urine were collected in a similar manner and assayed. The results are summarized in Fig. 1. These indicate that whereas the rate of excretion after oral administration was about the same for the sodium salt as for the free acid, the sodium salt was excreted at a much higher rate than the free acid when the compounds were given intraperitoneally.

The urinary excretion of 4-hydroxyisophthalic acid after the oral administration of graded doses of from 0.1 to 3.0 g./kg. was also measured, by cross-over tests with groups of four to five rats. The results, which are given in Table 3, show that with an increase in the dose the amount of 4-hydroxyisophthalic acid excreted in the urine during the first 24 hr. increases, although the percentage of the dose excreted decreases.

Estimation of 4-hydroxyisophthalic acid in rats' blood and alimentary canal

4-Hydroxyisophthalic acid (0.125 g./kg. of body wt.) was injected intraperitoneally into each of a number of rats which were killed after definite intervals. The 4-hydroxyisophthalic acid content of their blood was determined as described above. The results, given in Table 4, show that a maximum concentration is reached in about 15 min. and that after 30 min. the concentration falls rapidly, reaching a low level after 2 hr.

4-Hydroxyisophthalic acid was also administered by stomach tube, and the fate of the substance in the alimentary canal after an oral dose of 3 g./kg. was studied by assaying the 4-hydroxyisophthalic acid present in different sections of the digestive tracts of rats killed at various intervals after administration. The results, given in Fig. 2, show that large doses of 4-hydroxyisophthalic acid move very slowly through the alimentary tract and that absorption may well be too slow for the production of analgesia. The values used in Fig. 2 are the average from two experiments.

Excretion of 4-hydroxyisophthalic acid and its calcium salt by human subjects

In estimating the amount of 4-hydroxyisophthalic acid in human urine it was found that extraction with ether after acidification was desirable in order to reduce the amount of interference from the background colour. Two subjects each took orally a 0.66 g. dose of 4-hydroxyisophthalic acid and a 1.19 g. dose of the calcium salt (hexahydrate). In the first 24 hr. after taking the free acid, subject A excreted 15.2% of the acid, and subject B 13.7%. After ingestion of the calcium salt subject A excreted in the first 24 hr. 13.6% of the acid, and subject B 10.1%.

Table 3. Urinary excretion by rats of increasing doses of 4-hydroxyisophthalic acid administered by stomach tube

The weight of 4-hydroxyisophthalic acid administered to each group of four or five rats is shown in parentheses after the group letter at the top of each column. The recovery values are not corrected for the small blank obtained with the urine of untreated rats.

Time after administration (hr.)	Percentage and amount of 4-hydroxyisophthalic acid recovered from rats' urine after administration of (g./kg.)											
	0.1			0.3			1.0			3.0		
0-2	Group A (78.5 mg.)	Group B (98.5 mg.)		Group A (300 mg.)	Group D (740 mg.)	Group C (850 mg.)	Group A (850 mg.)	Group C (1770 mg.)	Group B (2580 mg.)	Group D (2805 mg.)		
	6.7% (5.3 mg.)	—		—	3.3% (24.3 mg.)	3.0% (25.2 mg.)	3.0% (25.2 mg.)	2.8% (49.1 mg.)	0.9% (22.6 mg.)	—		
2-4	13.2% (10.4 mg.)	—		—	3.8% (28.3 mg.)	3.7% (31.5 mg.)	3.7% (31.5 mg.)	2.1% (37.1 mg.)	2.9% (74.0 mg.)	—		
	—	—		—	—	—	—	—	—	—		
4-6	7.3% (5.7 mg.)	—		—	3.9% (28.8 mg.)	2.7% (23.1 mg.)	2.7% (23.1 mg.)	1.2% (20.6 mg.)	2.4% (63.2 mg.)	—		
	—	—		—	—	—	—	—	—	—		
6-24	14.0% (11.0 mg.)	—		—	17.2% (127.5 mg.)	18.7% (159.0 mg.)	18.7% (159.0 mg.)	16.7% (296.1 mg.)	17.0% (438.0 mg.)	—		
	—	—		—	—	—	—	—	—	—		
Total	41.2% (32.4 mg.)	42.1% (41.5 mg.)		31.1% (93.4 mg.)	28.2% (208.9 mg.)	28.1% (238.8 mg.)	28.1% (238.8 mg.)	22.8% (402.9 mg.)	23.2% (597.8 mg.)	21.2% (595.0 mg.)		

Table 4. Concentration of 4-hydroxyisophthalic acid in rats' blood after intraperitoneal administration

Time after injection (min.)	No. of rats	Mean of concns. $\pm$ s.e. of mean (mg./ml. of blood)	Range (mg./ml. of blood)
5	6	0.20 $\pm$ 0.026	0.12-0.30
15	6	0.24 $\pm$ 0.021	0.17-0.31
30	6	0.22 $\pm$ 0.035	0.13-0.33
60	7	0.089 $\pm$ 0.023	0.02-0.13
120	3	0.007	0.00-0.02
240	2	Nil	Nil

Fig. 2. Recovery of 4-hydroxyisophthalic acid from the alimentary tract of the rat after oral administration. $\bigcirc$ — $\bigcirc$, Total alimentary tract; $\triangle$ — $\triangle$, caecum + large intestine; $\times$ — $\times$, oesophagus + stomach; $\bullet$ — $\bullet$, small intestine.

Table 5. Urinary excretion of 4-hydroxyisophthalic acid by human subjects after oral administration

Period of collection (hr.)	Recovery (%)	
	Subject A	Subject B
0-9	8.2	4.1*
9-24	3.3	2.3
24-33	3.2	1.6
33-48	0.7	1.6
48-57	Not collected	0.4
57-72	0.8	0.8
Total	16.2	10.8

* Sample was incomplete.

In another experiment the urine was collected in six portions for 72 hr. after the ingestion of 0.66 g. of 4-hydroxyisophthalic acid. The results obtained are given in Table 5.

The most striking difference between the results with rats and human subjects is the relatively low recovery obtained in human urine and until experiments on faecal excretion have been carried out it is not possible to say whether the discrepancy is due to the drug being more completely metabolized or to its being excreted to a large extent in the faeces.

DISCUSSION

4-Hydroxyisophthalic acid is a substance of potential importance as an analgesic, with properties somewhat similar to those of acetylsalicylic acid. It was therefore desirable that a study should be made of its fate when administered to experimental animals and human subjects. The results now reported indicate that after oral administration of 10 mg. of the free acid to a rat between 30 and 40% of the dose was excreted in the urine within the first 24 hr., and very little more in the next 24 hr. Values of the same order were obtained when the calcium salt or a mixture of the acid and sodium bicarbonate were given orally, so that the free acid and its water-soluble salts are excreted in much the same way. Sodium bicarbonate was given with the free acid to determine whether or not this affected the excretion, since Alpen, Mandel, Rodwell & Smith (1951) observed that the addition of sodium bicarbonate to salicylic acid increased the amount of free salicylic acid excreted in human urine at the expense of salicyluric acid. With an oral dose of 20 mg. per rat of either the free acid or the sodium salt 40–50% was excreted in 24 hr. A much higher proportion of 4-hydroxyisophthalic acid was excreted after intraperitoneal administration of either the free acid or the sodium salt, namely 70–90%, and the rate of excretion was very much higher when the sodium salt rather than the free acid was injected, although there was little difference in the rates of excretion when the two substances were given by mouth. Although the proportion of the dose excreted in the urine decreased as the size of the dose given by mouth increased, the actual amounts eliminated by the kidney in 24 hr. may be considerable, exceeding 0.5 g./kg. of body weight with a dose of 3 g./kg.

When 4-hydroxyisophthalic acid was given to rats by mouth, 20–30% of a dose of 10 mg. per rat was excreted in the faeces in 24 hr. After the administration of a high dose, namely 3 g./kg., the amount present in the oesophagus and stomach fell steadily from the 80% that was present after 2 hr. to negligible amounts after 24 hr. A maximum concentration was reached in the small intestine at between 3 and 6 hr., and in the caecum and large intestine at about 16 hr. Substantial amounts were found in the last-named organs after 24 hr. It seems probable that the absence of analgesia in rats given 4-hydroxyisophthalic acid by mouth observed by Collier & Chesher (1956) is due to the slow rate of absorption, which prevents an adequate concentration of the substance being built up in the blood. It is interesting to compare this with the work of Smith (1954) and Smith & Irving (1955), who have shown that salicylate, on intraperitoneal

or intravenous injection, delays the emptying of the stomach in rats and that it acts systemically. The failure to demonstrate analgesia in rats after intraperitoneal administration of the sodium salt of 4-hydroxyisophthalic acid may, on the other hand, have been due to rapid elimination of the compound preventing the building up of an adequate concentration in the blood.

No evidence has been found that conjugated forms of 4-hydroxyisophthalic acid are excreted or that it is converted into other aromatic acids. It is known that, in man, salicylic acid is excreted partly as such, partly as salicyluric acid, partly as the glucuronide and to a very small extent as gentisic acid. Rats excrete much larger amounts of gentisic acid after administration of salicylic acid than do humans (Kapp & Coburn, 1942; Smith, 1949; Alpen *et al.* 1951; Smith, 1953).

SUMMARY

1. The absorption and excretion of 4-hydroxyisophthalic acid, a potentially important analgesic, were studied in rats and human subjects.

2. When 10 mg. of the acid per rat was administered orally, 30–40% was recovered in the urine within 24 hr. Similar results were observed after oral administration of equivalent amounts of the calcium salt or of a mixture of the acid and sodium bicarbonate.

3. On intraperitoneal injection of a suspension of the acid into rats 70–90% was excreted in the urine in 24 hr. About the same percentage of the compound was excreted in 24 hr. when the sodium salt was injected, but in this instance the rate of excretion was very much higher.

4. When 10 mg. of the acid per rat was administered orally, 20–30% was excreted in the faeces in 24 hr. The amounts of the compound present in various portions of the alimentary tract after oral administration of 3 g./kg. were estimated.

5. In human subjects 10–16% of a 0.66 g. dose of 4-hydroxyisophthalic acid was excreted in the urine within 24 hr.

6. In contrast to the results reported with salicylic acid, no evidence was obtained that 4-hydroxyisophthalic acid is excreted in the form of conjugates or hydroxylated derivatives.

We wish to thank the Directors of Allen and Hanburys Ltd. for permission to publish this work.

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A Comparative Study of Cerebrospinal Fluid and Serum Proteins in Multiple Sclerosis with Special Reference to the Lange Colloidal-Gold Reaction

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(Received 6 July 1955)

Positive colloidal-gold reactions in the cerebrospinal fluid of patients with multiple sclerosis have been reported as occurring in 29–81% of fluids examined by various investigators (Freedman & Merritt, 1949). Coagulation of the colloidal gold has been shown to be associated with the γ -globulin fraction (Kabat, Moore & Landow, 1942). The concentration of γ -globulin in cerebrospinal fluid was determined immunochemically by Yahr, Goldensohn & Kabat (1954), who report an increase in 66.5% of the multiple-sclerosis cases studied. The mechanism of several flocculation tests, including the colloidal gold at pH 7.8 and 6.6, was studied by MacLagan & Bunn (1947), using normal and hepatic serum fractions; they found that albumin and α - and β -globulins protected the colloidal gold and that γ -globulin brought about its coagulation.

Serum diluted to 0.03% protein gives a very strongly positive colloidal-gold reaction (Press, 1955), whereas a normal cerebrospinal fluid, having approximately the same concentration of albumin and globulins, gives a negative colloidal-gold reaction; this is suggestive of a qualitative difference between the protein fractions of cerebrospinal fluid and serum.

In the present study, the proteins of normal and pathological cerebrospinal fluids and normal sera have been compared both quantitatively and qualitatively. Cerebrospinal fluids and sera were analysed electrophoretically, and the percentage of γ -globulin in the cerebrospinal fluids was compared with the type of the colloidal-gold reaction. Albumin and α -, β - and γ -globulins have been isolated from sera and cerebrospinal fluid and the

effect of these fractions on the colloidal-gold reaction studied and their sedimentation constants determined.

Partial fractionations of cerebrospinal-fluid γ - and β -globulins were performed in the ultracentrifuge and the effect on the colloidal-gold reaction of the more slowly sedimenting fraction was examined. The chemical nature of this 'slow' component from both β - and γ -globulin was determined. Cerebrospinal-fluid γ -globulin was also fractionated with 18% sodium sulphate, and the sedimentation constant of the fractions and their effect on the colloidal-gold reaction were determined.

EXPERIMENTAL

Materials

Veronal buffer 1. This buffer (pH 8.6, $I=0.1$), which was used for micro-electrophoresis, contained 0.1M sodium veronal and 0.02M veronal.

Veronal buffer 2. This buffer (pH 8.6, $I=0.0375$), which was used for continuous paper electrophoresis, contained 0.0375M sodium veronal and 0.0075M veronal.

Phosphate buffer 1. This buffer (pH 7.4) was made by mixing 48 ml. of KH_2PO_4 (0.067M) and 202 ml. of Na_2HPO_4 (0.067M).

Phosphate buffer 2. This buffer ($I=0.2$, pH 7.45) contained 0.0142M- Na_2HPO_4 , 0.0025M- KH_2PO_4 and 0.1M-NaCl.

Sodium sulphate (27%, w/v) in phosphate buffer pH 7.8. For this solution 270 g. of anhydrous Na_2SO_4 , 6 g. of K_2HPO_4 and 0.488 g. of KH_2PO_4 were made up to 1 l. at 37°.

Dextran. Initially, a supply of 20% Dextran was available, approximate molecular weight 40 000 (kindly supplied by Dextran Co. Ltd.), but latterly Dextran was prepared by dialysing Intradex (a 6% w/v solution of Dextran in 0.9% NaCl) in cellophan tubing against daily changes of distilled water for 5 days and freeze-drying.

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