Removal of Acid by Trioctylamine from Samples for Microbiological Assay

By D. E. HUGHES and D. H. WILLIAMSON
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield 10

(*Received* 16 *September* 1950)

It was shown by Lester Smith & Page (1948) that certain cationic detergents, in particular the long-chain tertiary amines, may be used to transfer strong acids from an aqueous medium to organic solvents, e.g. chloroform. This procedure is based on the fact that the partition coefficient of the salts of the long-chain amines is greatly in favour of the organic phase. The most effective amine found was trioctylamine. By shaking aqueous hydrochloric acid with chloroform containing just over 1 equivalent of trioctylamine, more than 99 % of the acid was transferred to the chloroform phase. This method was applied by Lester Smith & Page to the removal of acid from protein hydrolysates, but has not been used before in the preparation of material for microbiological assays. The present paper reports a successful removal of trichloroacetic and hydrochloric acids from tissue extracts in which histidine was to be estimated by microbiological assay. The problem of histidine assay arose from joint studies, published elsewhere, on the source of the histamine found in tissue extracts (Wood, Hughes & Salvin, 1951).

EXPERIMENTAL

Microbiological assay of L-*histidine*. L-Histidine was assayed microbiologically with *Leuconostoc mesenteroides* P 60 according to Barton-Wright (1946), with a few modifications described below. Three samples containing different amounts of the material to be assayed were added to the medium (10 ml. final volume). The quantities were chosen to cover approximately the concentrations between 0·5 and 2·5 μg. histidine/ml.

The organism was maintained on malt agar stabs as described by Barton-Wright (1946), except that the inoculum for the assay was grown on the histidine assay medium with added histidine (50 μg./ml.) in place of the riboflavin assay medium. After 18 hr. growth the inoculum was centrifuged aseptically, washed twice in 0·9 % NaCl and diluted a 100-fold in saline; 0·1 ml. was added to each assay tube.

In the earlier experiments the response of the test organism was measured by titration of the acid formed after 3 days' incubation. Samples of 2·0 ml. from each of the assay tubes were titrated with 0·05 N-NaOH using bromothymol blue as described by McIlwain (1949). In most of the later experiments the response to histidine was measured turbidimetrically after 18–24 hr. incubation. The larger cells (4 × 1 cm.) of a Spekker absorptiometer were used when at least 7 ml. were available and the micro cells (0·3 × 1 cm.) when less material was available. Fig. 1 and Table 1 show the response to histidine measured by the two methods. It is evident from this curve that both methods are satisfactory, but the turbidimetric method was used in preference, as it was quicker.

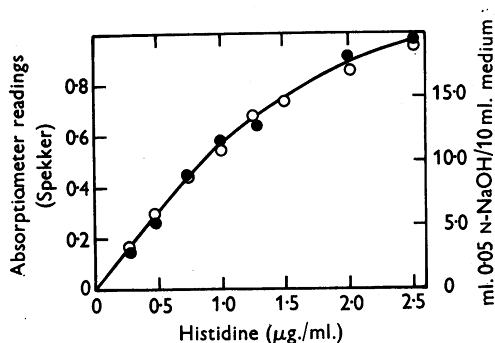


Fig. 1. Response of *Leuconostoc mesenteroides* to L-histidine. Experimental details are as described in the text (p. 487). Assay of growth: ●——●, acidimetric; ○——○, turbidimetric.

Histidine decarboxylase. *Clostridium welchii* (National Collection of Type Cultures, No. 6785) served as a source of histidine decarboxylase. The organism was grown as described by Gale (1947) except that the tryptic casein digest was replaced by 'Pronutrin' (a commercial casein hydrolysate marketed by Herts Pharmaceuticals Ltd.). The cells were collected by centrifuging, washed twice, and then resuspended in 0·9 % NaCl. The final suspension contained 20 mg. dry cells/ml. Otherwise the manometric determination of histidine was carried out as described by Gale (1947).

Chemicals. The tri-*n*-octylamine used in most of the work was a sample of 5 g. supplied by the courtesy of Dr E. Lester Smith, Glaxo Laboratories Ltd. This long-chain amine, as well as the others used by Lester Smith & Page (1948), became available commercially towards the latter part of the work from the British Hydrological Corporation, High Path, Abbey Road, London, S.W. 19. Samples of trioctylamine and methyl di-*n*-octylamine from this source were tested and found satisfactory. Carnosine nitrate was prepared from horse muscle and recrystallized four times from ethanol containing 40 % water.

RESULTS

Removal of trichloroacetic acid from blood and tissue filtrates

Freshly drawn venous blood (20 ml.) was pipetted into 30 ml. 10 % trichloroacetic acid and left for 1 hr. at 2°. The solution was then filtered through a Whatman no. 40 filter paper, and the precipitate was washed four times with 5 ml. 5 % (w/v) trichloroacetic acid. The volume of the combined filtrate and washings was adjusted to 100 ml. with water. Two portions, each of 25 ml., were pipetted into separating funnels and to one, histidine (75 μg.) was added. Each was then shaken with 50 ml. of a 5 % (w/v) solution of trioctylamine in chloroform saturated with water. After shaking, the emulsion was allowed to settle and the chloroform layer was separated. The pH of the aqueous layer was now 5·8–6·0 (glass electrode) against <1 before extraction. Thus the trioctylamine had removed the bulk of the acid. The removal of trichloroacetate was established by titration with silver nitrate, after acid hydrolysis at 100°.

A solution of histidine (75 μg.) in 25 ml. of 5 % trichloroacetic acid treated in the same way as the blood filtrate was also tested. The aqueous solutions were used directly for the microbiological assay of histidine.

Recovery of histidine was unsatisfactory where more than 1 ml. of the aqueous solution was used, which indicated the presence of interfering material, probably trioctylamine. Repeated extraction with chloroform reduced but did not completely abolish the inhibitory effect, but one extraction with chloroform followed by one extraction with ether removed the inhibitor completely in the case of blood (Table 1). In the case of other tissues two extractions with ether were needed to remove the inhibitory effect. The method finally adopted was as follows: the extract was treated with trioctylamine in chloroform as described above, and then extracted with 1 vol. of water-saturated ether. The funnels were rotated instead of shaken during the ether extraction in order to avoid emulsions. The bulk of the aqueous layer was transferred to a conical flask and aerated with moist air at about 30° until the smell of ether had almost disappeared (4–6 min.).

Table 1. *Recovery of histidine from blood and histidine solutions*

(For details of the method of assay see text, p. 487. Values in column *A* are histidine recoveries as measured by growth after 19–20 hr. incubation; values in column *B*, as measured by titration of the acid formed after 72 hr.)

Material added to the basal medium	Volume analysed (ml.)	Histidine (μg. total) Added	Recovered *A*	Recovered *B*
Histidine standard after treatment with trioctylamine and further extraction with $CHCl_3$ and ether	1	3·0	2·9	2·9
	2·5	7·5	7·0	6·5
	5·0	15·0	14·6	14·6
Filtrate from blood after treatment with trioctylamine $CHCl_3$ and ether	1	—	2·1	2·1
	2·0	—	4·0	3·8
	4·0	—	8·4	8·0

Handling of animal tissues. After removal from the animal, the tissue was cooled quickly to about 2° on ice, freed from water by blotting and weighed. The cooled tissue was cut quickly into small pieces with scissors and ground for about 30 sec. in a cold mortar with acid-washed sand. Cold 20 % (w/v) trichloroacetic acid (2·5 ml./g. tissue) was then added and the tissue was ground again. The mixture was filtered through a Whatman no. 40 filter paper and the solid residue washed three times with 5 % trichloroacetic acid; the combined filtrate and washings were made to a suitable volume, usually 0·01 g. tissue/ml.

Histidine in animal tissues

The results of the analysis of various tissues are shown in Table 2. Differences between duplicates on the same sample did not exceed 2 %. The figures for blood agreed fairly closely with those given by chemical estimations (Schwartz, Riegert & Bricka 1938). The figures for free histidine in extracts of muscle agree with those found recently in extracts of rat muscle by Schurr, Thompson, Henderson & Elvehjem (1950); there are no data in the literature on the histidine content of extracts of gastric mucosa.

Table 2. *Histidine content of blood, muscle and gastric mucosa*

(The extracts were made as described in the text. Trichloroacetic acid was removed by trioctylamine.)

Tissue	Histidine content (mg./100 g. wet wt.)* Before heating	After heating
Whole blood (cat)	2·2	2·0
Whole blood (rabbit)	1·16	1·15
Serum (rabbit)	1·40	—
Whole blood (human)	1·55	1·30
Serum (human)	1·60	—
Striated muscle (cat)	5·4	131
Striated muscle (cat)	5·2	129
Striated muscle (toad)	20·0	62
Gastric mucosa (cat)	2·0	1·7
Gastric mucosa (rabbit)	2·3	2·2
Gastric mucosa (toad)	5·5	—

* The results for blood and serum are expressed as mg. histidine/100 ml.

Release of histidine by heating acidified trichloroacetic extracts of tissues. Concentrated hydrochloric acid was added to the trichloroacetic extracts of tissue to bring the final concentration to 5 % (w/v)

hydrochloric acid and the solutions were heated for 1·5 hr. on the boiling-water bath as described by Code (1937). The histidine content of the solutions from cat muscle increased 20-fold and from toad muscle threefold: there was no increase in the extracts of blood, serum or gastric mucosa. A fivefold increase of histidine in extracts of rat muscle was found by Schurr *et al.* (1950), who ascribed the increase to the hydrolysis of carnosine. On this assumption the carnosine content of cat muscle calculated from the results in Table 2 would be about 200 mg./100 g. of tissue, which is in agreement with the values found by a chemical method (Hunter, 1925).

Carnosine solutions, if treated in the same way as the tissue extracts (1·5 hr. heating), showed 60–80% hydrolysis (Table 3). For complete hydrolysis 3·5 hr. heating were required. The yield of histidine from the hydrolysis of carnosine was the same in the presence of 5% hydrochloric acid, or 5% hydrochloric acid plus 5% trichloroacetic acid, which is noteworthy because the formation of histamine from histidine is increased by the presence of trichloroacetic acid (Åckerblom, 1941; Schmiterlöw, 1949).

Table 3. *Hydrolysis of carnosine by trichloroacetic acid and* HCl

(A carnosine solution (containing equivalent to 4·5 mg. histidine/ml.) in 5% trichloroacetic acid and 10% HCl was immersed in a boiling-water bath and samples taken at the times indicated. The acid was removed with trioctylamine as described in the text and the solutions assayed with histidine decarboxylase and by microbiological assay.)

Time (min.)	Histidine (mg./ml.) By decarboxylase	Histidine (mg./ml.) Microbiological assay
0	0	0
30	1·40	1·37
60	2·50	2·38
90	3·55	3·42
150	4·30	4·35
180	4·40	4·45

Recovery of trioctylamine

To recover the valuable trioctylamine, the chloroform phase containing the trioctylamine and the bound acid was shaken with sufficient 2N-sodium hydroxide to keep the pH of the aqueous layer above 12·0. The sodium hydroxide layer was separated and the chloroform washed twice with water. In this way relatively large quantities of acid could be removed with little loss of trioctylamine. The washed chloroform solution of trioctylamine did not contain histidine and could be used without further purification, for further experiments.

DISCUSSION

The trioctylamine method of Lester Smith & Page (1948), with minor modifications, has proved a satisfactory procedure for the removal of trichloroacetic and hydrochloric acids from tissue extracts in which histidine was to be estimated by microbiological assay. However, different tissues varied in their capacity to retain inhibitory quantities of trioctylamine. These can be removed by additional extractions with chloroform and ether, but special tests should be carried out with any new material to ascertain that trioctylamine has been effectively removed.

The principle of the method of Lester Smith & Page has recently been used by O'Keefe, Dolliver & Stiller (1949) to concentrate streptomycin; trioctylamine was replaced by a number of anionic detergents. Separation of some phosphoric esters has also been achieved by counter-current distribution with decylamine as a carrier in the organic phase (Plaut, Kuby & Lardy (1950).

SUMMARY

1. Histidine can be estimated by microbiological assay in aqueous extracts of tissues from which trichloroacetic acid has been removed by shaking with a chloroform solution of trioctylamine according to Lester Smith & Page (1948), provided that the extraction is followed by further extractions with chloroform and ether. These are necessary to remove inhibitory effects due to the retention of traces of trioctylamine in the aqueous phase.

2. Histidine values for the blood of cat, rabbit, and man and for gastric mucosa and striated muscle of rabbit, cat and toad are given.

3. Histidine in striated muscle increased about 20-fold on heating the solution with acid, presumably owing to the hydrolysis of carnosine.

The authors wish to express their thanks to Prof. H. A. Krebs, F.R.S., for his help and criticism, and to Dr E. Lester Smith for gifts of trioctylamine.

REFERENCES

Åckerblom, E. (1941). *Acta physiol. scand.* **2**, 378.

Barton-Wright, E. C. (1946). *Practical Methods for the Microbiological Determination of the Vitamin B Complex and Essential Amino-Acids.* London: Ashe Laboratories.

Code, C. F. (1937). *J. Physiol.* **89**, 259.

Gale, E. (1947). *Biochem. J.* **41**, vii.

Hunter, G. (1925). *Biochem. J.* **19**, 35.

Lester Smith, E. & Page, J. E. (1948). *J. Soc. chem. Ind., Lond.*, **67**, 48.

McIlwain, H. (1949). *Proc. roy. Soc.* B, **136**, 12.

O'Keefe, A. E., Dolliver, M. H. & Stiller, E. T. (1949). *J. Amer. chem. Soc.* **71**, 2452.
Plaut, G. W. E., Kuby, S. A. & Lardy, H. A. (1950). *J. biol. Chem.* **184**, 243.
Schmiterlöw, C. G. (1949). *Acta physiol. scand.* **19**, 130.
Schurr, P. E., Thompson, H. T., Henderson, L. M. & Elvehjem, C. A. (1950). *J. biol. Chem.* **182**, 29.
Schwartz, A., Riegert, A. & Bricka, C. R. (1938). *C.R. Soc. Biol., Paris*, **129**, 1159.
Wood, D. R., Hughes, D. E. & Salvin, E. (1951). *J. Physiol.* (Submitted for publication.)

Absorption and Excretion of Pheniodol Labelled with Radioactive Iodine

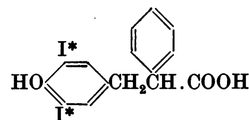
By AUDREY A. FREE, J. E. PAGE AND E. A. WOOLLETT
Research and Development Division, Glaxo Laboratories Ltd., Greenford, Middlesex

(*Received* 9 *September* 1950)

Pheniodol (α-phenyl-β-(4-hydroxy-3:5-diiodophenyl)-propionic acid) was introduced under the name 'Biliselectan' as a peroral cholecystographic agent by Dohrn & Diedrich (1940). After early clinical trials in Germany by Kleiber (1940), Grunke & Finger (1940), Lauer-Schmaltz (1940), Naumann (1941) and Rittweger (1941) and in Britain by Kemp (1943), and more extensive ones in America by Einsel & Einsel (1943), Marshall (1943), Wasch (1943), Dannenberg (1944), Bryan & Pedersen (1944), Hefke (1944), Ochsner (1944), Paul, Pohle & Benson (1944), Vaughan & Eichwald (1944), Unfug (1946) and Brewer (1947), it has been accepted (Harper, 1949) as the cholecystographic agent of choice. It is intended to include it in the Addendum (1951) to the British Pharmacopoeia, 1948.

The general pharmacology of pheniodol in animals has been discussed by Junkmann (1941), Modell (1942) and Howard (1948). However, except in Junkmann's paper, almost no information on the absorption and excretion of pheniodol by animals has been published. Junkmann's paper appeared when World War II was well under way; it is very difficult of access in this country and it may therefore be summarized here, especially as it has been quoted successively by several authors who do not all appear to have seen it. Junkmann, who was primarily interested in comparing pheniodol with tetraiodophenolphthalein, measured the iodine content of bile from biliary fistulas in two rabbits and examined the urinary excretion rate for rats and two men. He showed that most of the iodine in the urine was present as pheniodol by acidifying the urine, extracting into ether, evaporating the ether and recrystallizing the residue from benzene. The product was identified as pheniodol by its melting point and by formation of an insoluble calcium salt. A few distribution results have been mentioned by Howard (1948) and Salter, Karandikar & Block (1949).

The lack of metabolic data may be due to the difficulty of estimating organically bound iodine in biological fluids. The radioactive isotope labelling technique, however, is well suited to metabolic experiments, and we have used the method to study the absorption and excretion of pheniodol by rats and cats. Pheniodol labelled with radioactive iodine (^{131}I) was prepared by iodinating α-phenyl-β-4-hydroxyphenylpropionic acid with radioactive iodine.



Pheniodol labelled with ^{131}I.

In our first set of experiments, single doses of labelled pheniodol were given orally, subcutaneously or intravenously to rats, and the excretion of radioactive iodine was studied over a 3-day period. Single doses of the labelled compound were then injected intravenously or intraduodenally into anaesthetized cats, and the iodine blood levels and urinary and biliary excretions were followed over a 6 hr. period. By using a butanol-extraction procedure the proportion of radioactive iodine present in organic combination as pheniodol could be measured. The general experimental procedures were similar to those used for labelled thyroxine (Clayton, Free, Page, Somers & Woollett, 1950).

EXPERIMENTAL

Preparation of labelled pheniodol

A solution containing ^{131}I (1 mc.) in the form of iodide was transferred to a centrifuge tube containing NaI (10 mg.) and the volume of the solution was made up to 1 ml. Ether (1 ml.) and 1·0 ml. of 0·3% H_2O_2 followed by 4 drops of N-HCl were then added. The contents of the tube were