materials. Thus, as shown here, the unknown material may be treated with formaldehyde, dried under vacuum to remove the excess formaldehyde, and chromatographed. If the product has the same R_H value as compound A, is positive to the Pauly reagent, and contains no iodine, this should now constitute reasonable proof that it is 4(5)-iodohistidine. In the absence of a sample of 2-iodohistidine we can only deduce what would result from the treatment of this isomer with formaldehyde. Compound A containing an iodine substituent would result from the intramolecular condensation. On the other hand, since the electrophilic displacement of an iodine atom appears to take place faster, an intermolecular condensation could also occur to link the α-amino group of one MIH molecule to the C-2 carbon of another molecule through a methylene bridge. Since a reactive C-2 with an iodine substituent remains from this reaction, further intermolecular reactions can occur. Therefore, one can expect that polymers of various sizes which still contain one iodine atom at the terminal imidazole group would result. Thus, we conclude that the treatment of 2-iodohistidine with formaldehyde would show several spots on chromatography, each giving a positive iodine test.

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References

Ames, B. N., and Mitchell, H. K. (1952), *J. Am. Chem. Soc. 74*, 252.

Bensusan, H. B. (1966), *Arch. Biochem. Biophys. 115*, 77.

Bensusan, H. B., and Scanu, A. W. (1960), *J. Am. Chem. Soc. 82*, 4990.

Bowden, C. H., and Maclagan, N. F. (1954), *Biochem. J. 56*, vii.

Brunings, K. J. (1947), *J. Am. Chem. Soc. 69*, 205.

McDonald, C. C., and Phillips, W. D. (1963), *J. Am. Chem. Soc. 85*, 3737.

Neuberger, A. (1944), *Biochem. J. 38*, 309.

Ono, T., and Hirohata, R. (1956), *Z. Physiol. Chem.* 304, 77.

Pauly, H., and Arauner, E. (1928), *J. Prakt. Chem. 118*, 33.

Perlgut, L. E., and Wainio, W. W. (1966), *Biochemistry 5*, 608.

Identification of Mitochondrial Iodohistidine and Phosphoriodohistidine on a Sephadex G-10 Column*

L. E. Perlgut and W. W. Wainio

ABSTRACT: Monoiodohistidine and phosphoriodohistidine have been previously separated and identified on paper chromatograms [Perglut, L. E., and Wainio, W. W. (1966), *Biochemistry 5*, 608]; however, attempts at confirmation of this identification *via* ion-exchange column chromatography have been only partially successful. A Sephadex G-10 column produced separation and identification of the monoiodohistidine, phosphoriodohistidine, thyroxine, and triiodothyronine present in alcohol extracts of beef heart mitochondria Synthetic and mitochondrial monoiodohistidine, as eluted from the column, possessed identical ultraviolet absorption spectra; each contained labile iodine and produced comparable R_F values on paper chromatograms. ^{32}P-labeled mitochondrial extracts, when eluted from the column, produced a radioactive peak that coincided with that of synthetic phosphoriodohistidine.

We have previously reported separation and identification of mitochondrial monoiodohistidine (MIH)[1] and phosphoriodohistidine (PIH) by paper chromatography; however, ion-exchange column chromatography did not produce a positive identity (Perlgut and Wainio, 1966). We now wish to report separation and identification of both MIH and PIH, as well as thyroxine and triiodothyronine, on a Sephadex G-10 column.[2]

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[1] The following abbreviations will be used: monoiodohistidine, MIH; phosphoriodohistidine, PIH; adenosine triphosphate, ATP; thyroxine, T_4; triiodothyronine, T_3; FFCA, ferric ferricyanide–arsenious acid (Postmes, 1963); FFC, FFCA without the arsenious acid.

[2] Pharmacia Fine Chemicals, New Market, N. J.

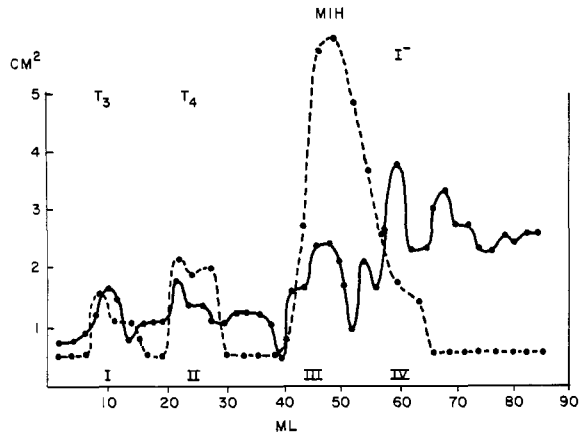

FIGURE 1: Chromatogram of a mixture of known iodine-containing compounds compared to the chromatogram of a mitochondrial extract. Beef heart mitochondria (20 mg of N) were incubated with a reaction mixture (containing β-hydroxybutrate) and extracted as previously described (Perlgut and Wainio, 1966). Measurement of volume of elulate was begun with the first fraction (2 ml) appearing after the fractions containing the Blue Dextran. The column was eluted with 0.1 M phosphate buffer, pH 6.7. ·····, standard mixture. ·—·—·, mitochondrial extract.

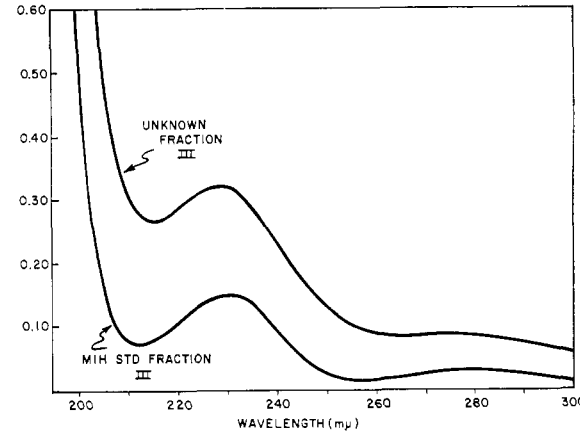

FIGURE 2: Comparison of the ultraviolet absorption spectrum of the unknown found in peak III (Figure 1) and synthetic MIH eluted in peak III (Figure 1).

A 1.5 × 90 cm column was prepared from 70 g of Sephadex G-10 suspended in 0.1 M phosphate buffer (pH 6.7 or 7.4). After washing the column for several hours with buffer solution, a 0.5-ml sample of Blue Dextran[2] solution was applied as a marker and, immediately afterward, 0.5–1.0 ml of the solution to be tested was added. Fractions (2 ml) were collected; the Blue Dextran usually appeared in about 4 ml of eluate after a void volume of 50 ml.

The iodohistidines were extracted from active beef heart mitochondria as previously described (Perlgut and Wainio, 1966); however, for these experiments, we used β-hydroxybutyrate, in place of 2-oxoglutarate–malonate, as substrate in the reaction mixture. In all cases, the mitochondrial extract was chromatographed on the same column with the same buffer as the standard mixture. To detect iodine-containing compounds, we spotted 10 μl from each fraction on filter paper and, after ultraviolet irradiation, sprayed the papers with the FFCA reagent (Perlgut and Wainio, 1964, 1966; Postmes, 1963). The optical density of the blue spots was measured in a densitometer equipped with a 600-mμ filter, and is reported as cm². Radioactivity was measured in a similar manner on filter paper using a Nuclear-Chicago actigraph II. In Figure 1, the FFCA intensity of the elution pattern of a mitochondrial extract is compared to the pattern from a mixture of known iodinated compounds. The presence of triiodothyronine (T_3, peak I), thyroxine (T_4, peak II), MIH (peak III), and iodide (peak IV) in the alcoholic mitochondrial extract is thus indicated. The rise of the base line after the iodide peak may be an indication of extensive tailing of iodide. This rise in base line was observed for some of the elution patterns of the standard iodoamino acid mixtures as well. Since the FFCA reagent is at least an order of magnitude more sensitive to iodide than it is to MIH, the size of the iodide peak does not indicate the relative abundance of iodide compared to MIH. Similarly, the small peak that appears at 55 ml on Figure 1 may be due to the addition of the beginning of the iodide peak to the tail of the MIH peak. Ninhydrin-positive material appeared between 5 and 40 ml as a large irregular peak, probably phospholipid. Histidine was included in the control mixture and was detected with ninhydrin, at 35 ml. In our hands the FFCA reagent was completely insensitive to histidine and lipids. The ultraviolet absorption spectrum of the pooled fractions of standard peak III (MIH) and of sample peak III is shown in Figure 2. (Previous determination of the absorption spectrum of MIH was not possible, since we lacked a pure sample.) The identity of unknown peak III with standard peak III was thus indicated. Paper chromatography of the various peaks of the unknown and known samples produced only iodide for each, in two different chromatographic systems (Figure 3). The reason for these results became clear when we chromatographed on paper the standard mixture with and without addition of a portion of the buffer eluted from the column. The result was a large increase in iodide and a sharp decrease in the amount of T_4, T_3, and MIH detectable. Evidently, some material eluted from the column catalyzed the deiodination reactions on paper. The net result of the paper chromatography, in two solvent systems, was confirmation that each of the FFCA-positive peaks did contain an iodinated compound, since iodide was positively identified in each (Figure 3).

On reconsidering the results of the paper chromatography experiment, it occurred to us that perhaps our technique was at fault, and that it may have been possible to reduce the deiodination reaction by sub-

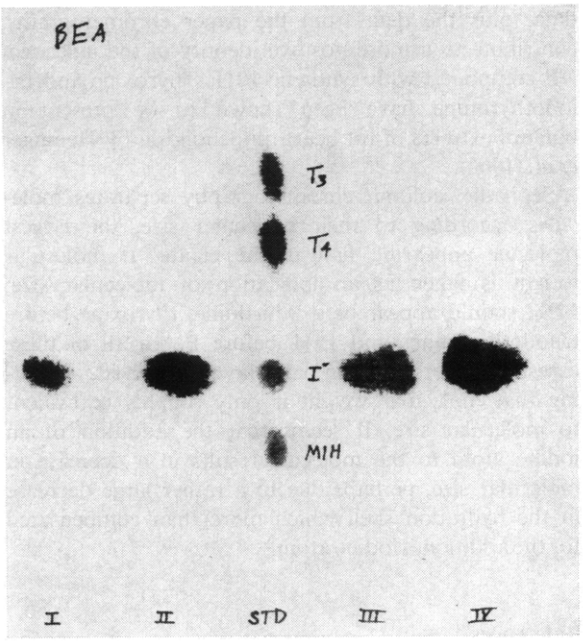

FIGURE 3: Chromatograph of the FFCA-sensitive peaks recovered from a Sephadex column as shown in Figure 1. The applied samples (200 μl) were dried on the filter paper with a stream of heated air. The standard mixture (STD) did not require heat since only 10 μl was used. Solvent system (BEA): *n*-butyl alcohol–95% ethyl alcohol–concentrated ammonium hydroxide–water (10:2:2:2).

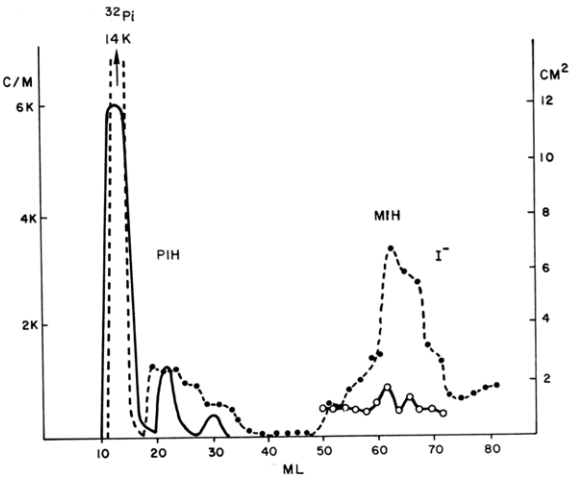

FIGURE 4: Identification of PIH on Sephadex G-10 column. Beef heart mitochondria containing 5 mg of N were treated and extracted as previously described. -------, radioactivity of standard mixture, 10-μl samples. ·-·-·-·-·, intensity of FFCA color of standard mixture, 10-μl samples. ———, radioactivity of mitochondrial extract, 10-μl samples. O--O--O, intensity of FFCA color of mitochondrial extract, 50-μl samples.

TABLE I: R_F Values of Sephadex G-10 Column Eluates, Fraction 33 of Both Synthetic MIH, and a Mitochondrial Extract.[a]

	R_F Values			
Solvent System	Fraction 33 MIH	Fraction 33 Unknown	MIH Std	I^- Std
BAC	0.09–0.12	0.10–0.13	0.08–0.10	0.16–0.18
BEA	0.15–0.22	0.14–0.18	0.19–0.24	0.35–0.37

[a] The solutions to be tested were applied to the filter papers without the use of heat. Solvent systems used were BAC: *n*-butyl alcohol–acetic acid–water (78:5:17) and BEA: *n*-butyl alcohol–95% ethyl alcohol–concentrated ammonium hydroxide–water (10:2:2:2). The papers were developed for 18 hr in dim light.

stituting a stream of cold air for the heated air used in drying the samples as they were applied to the filter paper. New samples of both synthetic MIH and the mitochondrial extract were again chromatographed on a fresh Sephadex column and, this time, the peaks were detected by measuring their absorbance at 230 mμ. Fraction 33 of the extract produced a jump in optical density as did fractions 32 and 33 of the MIH standard. This method of detecting the peaks eliminated the interference by inorganic iodide with the MIH peak, as detected by the FFCA method. Both fractions 33 were then evaporated to dryness, *in vacuo*, and redissolved in 0.5 ml of 33% ethanol. These solutions (100 μl) were then spotted on filter papers, using a stream of cold air to dry the spots. After chromatography, the papers were irradiated with ultraviolet light and sprayed with the FFCA reagent, as before. The results (Table I) confirmed that fraction 33 of the mitochondrial extract contained an iodine compound that migrated with the same R_F values as fraction 33 of the MIH sample. In order to eliminate the possibility that we may have detected an FFCA-sensitive compound that does not contain iodine, we sprayed (after ultraviolet irradiation) a duplicate set of chromatograms with FFC (without arsenious acid). Postmes (1964) and Row *et al.* (1966) have shown that iodoamino acids are not sensitive or weakly sensitive to FFC, while noniodine compounds that are sensitive to FFCA are equally sensitive to FFC. The paper treated with FFC produced only a very faint crescent above the origin and no other spots detectable by sight or with a densitometer.

The data from the heat-applied chromatograms (Figure 2) plus the above data from the cold-applied chromatograms (Table I) confirmed that we had separated an iodine-containing compound with the same mobility as MIH through Sephadex G-10, with the same R_F values on paper as MIH, and with the same ultraviolet absorption spectrum as MIH.

Radioactive mitochondrial PIH was prepared by incubating beef heart mitochondria with $^{32}P_i$, as described previously (Perlgut and Wainio, 1966). The extract was applied to the Sephadex G-10 column and eluted with 0.1 M phosphate buffer, pH 7.4. The comparison of the elution pattern of the mitochondrial extract with the pattern of a known sample containing PIH, MIH, and $^{32}P_i$ appears in Figure 4. The chromatogram of the extract produced a second radioactive peak that coincided with the synthetic PIH peak, and was well separated from $^{32}P_i$ peak. In the chromatogram of the standard mixture, ATP appeared in the fraction immediately before the $^{32}P_i$ peak; the chromatogram of the sample indicated that [^{32}P]ATP added on to the $^{32}P_i$ peak and the result was a slight broadening of the peak. Aliquots of the fractions between 50 and 70 ml were applied to filter paper and sprayed with FFCA reagent as before, producing a peak that coincided with the synthetic MIH peak. As expected, this peak was much lower than the one in Figure 1, indicating that a substantial fraction of the mitochondrial MIH had been converted to PIH. The fact that PIH appeared in substantially the same fractions as mitochondrial triiodothyronine made the use of the FFCA reagent valueless as a PIH detector.

In our previous report we had shown that the only alkali-stable, acid-labile ^{32}P compound present in our mitochondrial extract migrated with the same R_F value as synthetic PIH on paper, contained iodine, and, on acid hydrolysis, produced MIH (Perlgut and Wainio, 1966). The alcohol extract used here was prepared as before, and since the only unknown ^{32}P peak co-migrated with synthetic PIH, we feel that these data, plus the data from the paper chromatograms, contribute to a more positive identity of the unknown ^{32}P compound with synthetic PIH. Thyroxine and triiodothyronine have been shown to be present in butanol extracts of rat heart mitochondria by Heninger *et al.* (1966).

Sephadex column chromatography separates molecules according to their molecular size, the largest molecule appearing first in the eluate. If molecular weight is taken as an indication of molecular size, MIH should appear before histidine, thyroxine before triiodothyronine, and PIH before P_i. In all of these cases, the reverse elution pattern was observed, indicating that molecular weight is only roughly equivalent to molecular size. It seems that the addition of an iodine atom to the molecule results in a decrease in molecular size, perhaps due to a rather large decrease in the hydration shell which more than compensated for the additional iodine atom.

References

Heninger, R. W., Larson, F. C., and Albright, E. C. (1966), *Endocrinology 78*, 61.

Perlgut, L. E., and Wainio, W. W. (1964), *Biochem. Biophys. Res. Commun. 16*, 227.

Perlgut, L. E., and Wainio, W. W. (1966), *Biochemistry 5*, 608.

Postmes, T. (1963), *Acta Endocrinol. 42*, 153.

Postmes, T. (1964), *Clin. Chim. Acta 10*, 581.

Row, V. V., Volpé, R., and Ezrin, C. (1966), *Clin. Chim. Acta 13*, 666.