A METHOD FOR THE DETERMINATION OF ERGOTHIONEINE IN BLOOD*

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(Received for publication, August 12, 1952)

One of the difficulties associated with research on ergothioneine has been the lack of completely reliable methods for the quantitative determination of the compound in blood and other tissues. Ergothioneine was originally discovered to be present in the red blood cell by Benedict *et al.* (1, 2) and by Hunter and Eagles (3, 4), some 25 years ago, because of its reducing action on the arsenophosphotungstate reagent devised by Benedict for the determination of uric acid, and the earlier quantitative methods (5, 6) for ergothioneine were based on the use of this or similar reagents. The non-specificity of the uric acid reagents left much to be desired. In 1928 Hunter (7) reported that the coupled product of ergothioneine with diazotized sulfanilic acid yields a characteristic magenta color in the presence of strong alkali. This reaction, which is highly sensitive for ergothioneine, shows excellent specificity (8, 9) and appears to be the most suitable basis for a quantitative method. The method proposed by Touster (10), based on the oxidation of the sulfur of ergothioneine to sulfate by means of bromine, suffers from lack of specificity and non-quantitative recovery of ergothioneine added to blood.

The difficulties involved in applying the diazo reaction to the analysis of ergothioneine in natural materials are due to the fact that many substances (*e.g.*, tyrosine) yield yellow to orange colors with the diazo reagent and thereby mask the magenta color given by ergothioneine, while other substances (*e.g.*, glutathione) inhibit the color formation from ergothioneine itself. Compounds of both classes occur in blood filtrates. The usefulness of the diazo reaction is therefore dependent on the availability of an effective procedure for the removal of the interfering substances before the diazo reaction is applied. The difficulties in developing such a procedure are complicated by the variations in the amounts of interfering substances present, not only in blood from different species, but even in blood from different individuals within the same species. Hunter's original diazo method is deficient in this respect. Modifications of the method, recently published by Hunter (11) and by Lawson, Morley, and Woolf (12), are attempts to overcome this difficulty. Hunter uses basic lead acetate to re-

* This work was aided by a grant from the Nutrition Foundation, Inc.

move interfering substances from whole blood filtrates; however, occasional interferences were still noted (11, 13). To eliminate remaining interfering substances, Lawson and coworkers remove plasma and in addition precipitate ergothioneine with iodobismuthous acid; some ergothioneine is lost owing to incomplete precipitation, and the sensitivity of the method is thereby reduced. In our experience with the Hunter and Lawson methods, not all blood samples tested have yielded satisfactory colors.

In the method reported here, the diazo reaction is carried out on blood filtrates from which interfering substances have been removed by means of an ion exchange resin and organic solvent extraction. Of several dozen mammalian blood samples so far analyzed by the method, none has given poor colors. Further advantages of the method include increased sensitivity which permits greater certainty in the determination of low ergothioneine levels and applicability to the analysis of certain other tissues for ergothioneine.

EXPERIMENTAL

Determination of Ergothioneine in Pure Solution[1]—The procedure of Hunter (11) was modified to increase the sensitivity of the method by the use of larger volumes of test solution in conjunction with smaller volumes of more concentrated reagents. The sodium carbonate-sodium acetate buffer solution of Hunter was replaced by a sodium carbonate-sodium citrate mixture; the citrate was found to be useful in minimizing interference from traces of iron. Several substances were tested for possible production of extraneous color or inhibition of color formation from ergothioneine. Histidine, tyrosine, thiolhistidine, and carnosine gave yellow to orange colors. Poor colors were obtained from ergothioneine in the presence of iron, copper, or ammonium ions; iodide, fluoride, sulfite, or sulfur; ascorbic acid, cysteine, or glutathione; bilirubin, creatinine, digitonin, alcohols, phenols, or ketones. Optical densities were measured in a spectrophotometer at 540 mμ, rather than 510 mμ as used by Hunter, since light absorption at the longer wave-length is less affected by the presence of such substances as histidine and tyrosine, while the sensitivity toward ergothioneine is not appreciably changed.

The relationship of optical density to ergothioneine concentration is a straight line function (Fig. 1). Approximately 30 γ of ergothioneine produce the maximum optical density readable with fair accuracy; this sensitivity is appreciably greater than the sensitivity attained by Lawson and coworkers, and is somewhat greater than that given by the method of Hunter.

[1] The ergothioneine was isolated from ergot by Mr. C. C. Otken in this laboratory by a modification of the method of Hunter, Molnar, and Wight (14).

Preparation of Blood Samples—As earlier investigators have observed, ergothioneine may gradually disappear from blood which is allowed to stand either at room temperature for a few hours or at refrigerator temperatures for several days. Whenever possible, therefore, samples have been processed soon after collection. Potassium or sodium oxalate, sodium citrate, and heparin were found to be suitable anticoagulants. In this work heparin has been used routinely. Because plasma contains substances which give yellow or orange colors in the test (11), and contains no

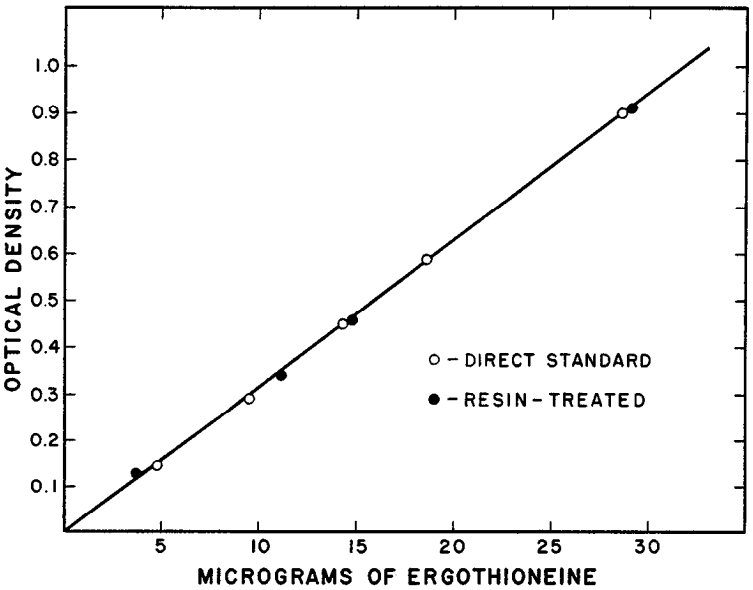

FIG. 1. Comparison of diazo color intensities given by solutions of pure ergothioneine with those given after treatment of trichloroacetic acid solutions of ergothioneine with IRA-410 ion exchange resin.

detectable ergothioneine, it was removed from the cells. This was done most conveniently by delivering the heparinized blood into several volumes of isotonic saline solution and centrifuging. We have found no loss of ergothioneine from the cells in this procedure (*cf.* also Fraser (15)). The washed cells may be stored for several days in the frozen state.

Blood Protein Precipitation—Varying recoveries of ergothioneine were obtained by earlier investigators with such protein precipitants as tungstic acid, molybdic acid, and tungstomolybdic acid (*cf.* Behre (16)). We found little or no ergothioneine in blood filtrates prepared with the above acids or with sulfosalicylic acid, metaphosphoric acid, trichloroacetic acid, or zinc hydroxide. We have discovered, however, that the addition of various reducing agents to the laked cells, prior to precipitation of the protein

with trichloroacetic acid, gives better recoveries of ergothioneine in the filtrate. The most satisfactory procedure for analytical purposes is treatment of the laked cells with glutathione and sodium hydrosulfite (dithionite); the use of both reagents is essential for maximum yields of ergothioneine, as shown in Table I. After this treatment, precipitation of the proteins with trichloroacetic acid results in quantitative recovery of ergothioneine in the filtrate. This procedure was found to give filtrates which contained less protein and other interfering substances than filtrates prepared by the heat coagulation method used by Hunter.

Removal of Interfering Substances—The removal of interfering substances from the blood filtrates without loss of ergothioneine presented the major

TABLE I

Effect of Addition of Sodium Hydrosulfite and Glutathione to Cells from 1 Ml. Aliquots of Rat Blood Prior to Protein Precipitation with Trichloroacetic Acid

Hydrosulfite	Glutathione	Color	Ergothioneine
mg.	*mg.*		γ
None	None	Yellow	
5	"	Magenta	15.2
15	"	"	15.4
25	"	"	15.6
50	"	"	12.6
25	5	"	22.5
25	10	"	24.6
25	15	"	26.0
25	20	Salmon-pink	27.5
25	30	"	26.0

difficulty of the analytical procedure. The use of basic lead acetate, followed by precipitation of the lead with NaH_2PO_4, as used by Hunter for the removal of glutathione, in our hands resulted in small losses of ergothioneine when this procedure was applied to pure ergothioneine solutions. Adsorption of ergothioneine on Norit, Lloyd's reagent, silica gel, or alumina, followed by elution with various reagents, gave poor to fair recoveries. Alumina columns showed the greatest promise, but it was not possible to achieve quantitative separation of ergothioneine and glutathione on columns of a size suitable for routine analyses. Several ion exchange resins were investigated. Satisfactory results were finally obtained with IRA-410, a basic resin which is stated by the manufacturer to be effective in removing traces of hydrogen sulfide from aqueous solutions. Several salts of the resin were studied; the most promising was a mixture of the chloride and carbonate salts obtained by treating the commercially available chloride form with sodium carbonate. This material, under controlled

conditions, removed glutathione and other interfering substances from blood filtrates with no loss of ergothioneine. The amount of resin, the time interval of shaking, and the moisture content of the resin influenced to some extent the recoveries obtained. Ergothioneine solutions which were treated with thoroughly dried resin gave recovery values in excess of the theoretical, owing presumably to preferential absorption of water by the resin. Moist resin was therefore used in the determinations. Prolonged shaking of ergothioneine solutions with the resin resulted in the loss of ergothioneine, as did the use of excessively large amounts of resin. None of these factors was found to be highly critical, and satisfactory recoveries were uniformly obtained with resin prepared and used as described below.

Blood filtrates prepared in the above manner occasionally yielded colors in the diazotization procedure which contained a yellow component not present when ergothioneine solutions were used. It was found that the material responsible for the yellowing could be removed by extraction of the filtrates with chloroform. The extraction procedure was most effective when it was applied to the filtrates before treatment with resin. During the extraction a small amount of solid material separated; both this solid and the material in the chloroform extract gave a yellow color in the diazotization reaction. The chloroform-extracted filtrates, when treated with resin and then carried through the diazotization procedure, gave the magenta color typical of pure ergothioneine.

Reagents—

Trichloroacetic acid, reagent grade, redistilled in an all-glass apparatus; used as a 35 per cent solution in water. The amount of trichloroacetic acid used in the protein precipitation is close to the minimum required for satisfactory precipitation, in order that excessive amounts of resin will not be required later to remove the acid. The solution is kept refrigerated and replaced every week. The use of solutions which have deteriorated results in poor recoveries of ergothioneine from the subsequent resin treatment.

Chloroform, reagent grade, washed six times with equal volumes of distilled water, and stored in a dark glass bottle; washed once with water on the day it is used.

Sodium hydrosulfite, powdered (Mallinckrodt).

Glutathione (Schwarz Laboratories, Inc., New York), aqueous solution, 35 mg. per ml., prepared just before using. Some commercial samples of glutathione appeared to contain impurities which caused interference in the diazo reaction. The specified brand has worked satisfactorily.

Ion exchange resin, Amberlite IRA-410 (The Resinous Products Division, Rohm and Haas Company, Philadelphia). Prepared by shaking 500 gm. of the commercial moist chloride salt with 2 liters of 5 per cent Na_2CO_3

for 3 hours, washing with distilled water until free from alkali, and collecting on a funnel with the aid of suction. When no more liquid passed the filter, the moist resin was transferred to a container, with a closely fitting cover, to preserve the moisture content.

Alkaline buffer solution. Sodium carbonate, reagent grade, anhydrous (200 gm.) and sodium citrate dihydrate (57 gm.) were dissolved in approximately 800 ml. of water with heating. The solution was cooled, filtered by gravity, and diluted to 1 liter. The solution is stored in polyethylene containers and is refiltered if sediment forms.

Sulfanilic acid, reagent grade, 0.2 per cent solution in 1 per cent HCl.

Sodium nitrite, reagent grade, 8 per cent solution in water.

Diazotized sulfanilic acid solution. 1 volume of cold $NaNO_2$ solution added to 10 volumes of cold sulfanilic acid solution, mixed, and allowed to stand 10 minutes before using, and kept in an ice bath. Prepared fresh on the day it is to be used.

19 N sodium hydroxide, prepared from reagent grade NaOH, filtered through a sintered glass funnel, and stored in polyethylene containers. The solution should be refiltered if subsequent cloudiness develops.

Procedure

1 ml. of heparinized blood is delivered into a 12 ml. graduated centrifuge tube containing 10 ml. of 0.9 per cent NaCl solution. The blood is thoroughly mixed with the salt solution and the mixture is centrifuged. As much as possible of the diluted plasma is removed. Water is added to the cells to the 1.0 ml. mark, and the mixture is stirred with a glass rod to facilitate laking. Then 0.5 ml. of a freshly prepared solution of glutathione containing 35 mg. per ml. is added and the solutions are well mixed. Approximately 25 mg. of solid sodium hydrosulfite are added and the mixture is stirred. The tube contents are mixed with 5.0 ml. of water, and the proteins are precipitated by the addition of 0.5 ml. of 35 per cent trichloroacetic acid. The well stirred mixture is centrifuged, and the supernatant solution is poured into a small separatory funnel and shaken with 15 ml. of washed chloroform. The chloroform phase and any solid material are discarded, and the aqueous layer is transferred to a glass-stoppered tube of 15 ml. capacity. Approximately 4 ml. (about 2.4 gm.) of moist ion exchange resin are added. The tube is stoppered and shaken mechanically for 10 minutes. The solution is separated from the resin by gravity filtration through a funnel containing a small plug of absorbent cotton.

A 3 ml. aliquot of the chloroform- and resin-treated filtrate is transferred to a soft glass test-tube, 18 mm. outside diameter, which has been calibrated for use in the photometer. The tube is placed in an ice bath, 0.5 ml. of alkaline buffer solution is added, and the solutions are mixed. To the cold

solution 0.5 ml. of diazotized sulfanilic acid is added, and the solutions are mixed and allowed to stand in the ice bath for 45 seconds. Then 1.0 ml. of 19 N NaOH is added rapidly, and the solutions are well mixed. The solution is allowed to stand at room temperature until it is free from gas bubbles, and then the optical density at 540 mμ is determined in a Coleman junior spectrophotometer. It was necessary to modify the cuvette holder to permit reading 5 ml. volumes in the 18 mm. test-tubes. The instrument scale was adjusted to zero optical density with a blank solution prepared by substituting 3 ml. of water for the test solution. A blank prepared by shaking 7 ml. of 2.5 per cent trichloroacetic acid with resin and then treating 3 ml. of the resulting filtrate with the diazo reagents gave a negligible increase in reading over the direct blank prepared without resin treatment.

A calibration curve was obtained by the use of 3 ml. portions of pure ergothioneine solutions, of varying concentrations, in the diazotization procedure. Fig. 1 shows the results obtained when the ergothioneine solutions were used directly in the diazo reaction, and also when solutions were first prepared in 2.5 per cent trichloroacetic acid, treated with resin, and then diazotized. The curves show essentially 100 per cent recoveries at all levels of ergothioneine concentration. In routine analyses, a 3 ml. portion of a standard ergothioneine solution containing 15 to 20 γ of ergothioneine is diazotized with each series of blood samples.

Since three-sevenths of the ergothioneine in 1 ml. of blood is used in the final color reaction, the values expressed in Fig. 1 are multiplied by 7/3 to give the results in terms of micrograms of ergothioneine per ml. of blood. The calibration curve is therefore usable over a range of ergothioneine concentrations up to approximately 7 mg. per 100 ml. of blood. This range includes the large majority of blood samples we have examined. Specimens with higher levels can be determined either by the use of smaller amounts of blood filtrate in the diazo reaction or by using less blood initially. If 0.5 ml. of blood is used, the amounts of glutathione and hydrosulfite are decreased proportionately, but the amounts of the other reagents remain unchanged. It is of interest that 0.5 or 1.0 ml. samples of the same blood yield ergothioneine values which are proportional to the amount of blood used. Hunter (11) occasionally found decreased ergothioneine values when increased amounts of blood filtrate were used, owing to incomplete removal of inhibiting substances which show disproportionately greater effects at higher concentrations. Touster and Yarbro (17) encountered the reverse phenomenon, in which enhanced values were obtained with the use of increasing amounts of blood filtrate.

Recoveries of Ergothioneine Added to Blood—Ergothioneine was added to red blood cells from which the plasma had been removed, and the mixture was carried through the procedure as described above. Some typical re-

covery data for human blood are given in Table II and for other species in Table III.

Blood Ergothioneine Levels—The ergothioneine values obtained on a series of normal adult humans are shown in Table IV. In these cases the procedures of Hunter (11) and of Lawson and coworkers (12) were applied to the same blood samples. The results obtained with all three methods

TABLE II

Recoveries of Ergothioneine Added in Varying Amounts to Cells from 1 Ml. Aliquots of Human Blood

Subject	Ergothioneine, γ per ml. blood			
	Original	Added*	Found	Calculated
M	18.6	19.2	38.5, 37.0	37.8
	18.6	38.4	56.2, 55.0	57.0
	18.6	58.5	74.0, 75.0	77.1
P	30.6	20.6	52.1, 53.2	51.2
	30.6	41.2	71.0, 70.0	71.8
	30.6	61.6	93.5, 96.0	92.2

* The duplicate determinations were carried out by adding the indicated amounts of ergothioneine to separate aliquots of cells.

TABLE III

Recoveries of Ergothioneine Added to Cells from 1 Ml. Aliquots of Animal Blood

Species	Ergothioneine, mg. per 100 ml. blood			
	Original	Added	Found	Calculated
Rat	3.2	2.2	5.4	5.4
"	3.2	4.5	7.8	7.7
Rabbit	4.1	2.7	6.5	6.8
Cat	1.6	2.2	3.8	3.8
Dog	3.7	2.2	6.0	5.9
Ox	2.3	1.7	3.8	4.0
Pig	8.6	4.5	13.2	13.1

were in substantial agreement when the quality of the colors obtained permitted comparison; in some samples with low ergothioneine levels, we were not able to obtain magenta colors by the Hunter or Lawson methods. In our limited experience with the Lawson method, we were not completely successful in controlling the losses of ergothioneine which occur during the precipitation with iodobismuthous acid; this may explain some of the divergences in results between the present method and that of Lawson.

While the present method worked satisfactorily on blood from the species indicated in Table III, attempts to apply it to chicken blood resulted in

colors which contained an appreciable amount of yellow or orange component. Considerable improvement in the color was obtained by using picric acid and trichloroacetic acid in the protein precipitation. This was accomplished conveniently by the substitution of 2 ml. of saturated picric acid solution for 2 ml. of the 5 ml. of water used to dilute the laked cells before precipitation with trichloroacetic acid. It was also found helpful to increase the volume of resin to approximately 5 ml. With this modified procedure some samples of chicken blood yielded magenta colors in the diazo test, but in other samples the interference was not completely removed. We have not examined other species of nucleated cells.

TABLE IV

Comparison of Methods for Determination of Blood Ergothioneine Levels in Normal Human Subjects

Subject	Ergothioneine, mg. per 100 ml. blood*		
	Method of Hunter	Method of Lawson *et al.*	Present method
BG	2.0	2.3	2.0
JP	2.0		1.8
CR	2.5	1.6	2.9
DA			1.5
RL	3.1	3.2	4.2
MC			1.7
MA	2.1	1.6	1.8
DM		1.9	1.9

* Values are given only for determinations in which satisfactory magenta colors were obtained.

DISCUSSION

The fact that the addition of reducing agents to laked blood cells will prevent loss of ergothioneine during the subsequent precipitation of proteins with trichloroacetic acid is of considerable interest with regard to the state of ergothioneine in the red cell. One possible explanation is that ergothioneine may be bound to red cell protein in a linkage which is split by glutathione and hydrosulfite, or by heat coagulation in dilute acid solution. Further work is required to settle this point. Whatever the explanation, for the present purposes it is of interest that the ergothioneine levels obtained by both of these quite different procedures are consistent. Such agreement suggests that the ergothioneine values found are a true measure of the ergothioneine content of the red cell. The excellent recoveries of ergothioneine added to cells lend weight to this supposition.

The use of glutathione as a reagent in this procedure may appear undesirable in view of the fact that glutathione inhibits the color produced by

ergothioneine. However, the ion exchange resin is quite effective in removing the necessary amounts of added glutathione. The only other reagent which we found to be completely effective in replacing the glutathione-hydrosulfite mixture is 2,3-dimercaptopropanol (BAL); this substance also caused interference with the diazo color and was not readily removed from the blood filtrates by the resin and chloroform treatment.

One advantage of the method described is the greater sensitivity at low ergothioneine levels. We have had occasion to examine animal bloods which contained no demonstrable ergothioneine. In these cases the diazo color was a pale straw-yellow. This slight amount of residual interference is not noticeable in the presence of low ergothioneine concentrations, and apparently does not affect the results to a significant extent.

Tissues and fluids other than blood were examined to determine the limitations of the method. Samples of human umbilical cord blood and menstrual blood gave little interfering color and were found to contain ergothioneine. Blood platelets and leucocytes, saliva, urine, and semen gave yellow to orange colors. Homogenized tissues from various organs of the rat yielded yellow to brown colors. When picric acid was used in conjunction with trichloroacetic acid to precipitate proteins, interfering substances were effectively removed from liver, heart, and kidney homogenates. This method permits the determination of ergothioneine in these tissues with much greater certainty than can be accomplished by the procedure described by Heath and coworkers (18), in which the tissues from rats to which ergothioneine had been administered were compared with tissue "blanks" from rats which had received no ergothioneine. Tissues which we examined other than the three mentioned gave too much interfering color to permit an accurate determination of ergothioneine.

SUMMARY

A method for the quantitative determination of ergothioneine in blood has been devised, based on the treatment of laked red cells with hydrosulfite and glutathione, precipitation of proteins with trichloroacetic acid, and removal of interfering substances by extraction with chloroform and adsorption on an ion exchange resin. Ergothioneine is then determined by a modification of the diazotization reaction previously described by Hunter. All mammalian bloods examined have given satisfactory colors and good recoveries of added ergothioneine. The method is applicable to the analysis of certain tissues for ergothioneine.

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J. Biol. Chem. 1953, 200:275-285.