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Multifunctional Reagent for Erythrocytes

Abstract

The present invention relates to new reagents and methods for the treatment, and in particular the lysis of erythrocytes.

5 The invention provides a multifunctional reagent for erythrocytes wherein it contains a quantity sufficient to produce the lysis of erythrocytes or the sphering of erythrocytes in such a way that they can be detected by a cytometer or an automatic counting device, of a carbamate or of an agent inducing the formation by the erythrocytes, from carbonate and from a nitrogenated heterocycle or ammonium ions, of a carbamate combined with the absorption of CO_2 by said erythrocytes.

10 The invention also provides a process for the lysis or the sphering of the erythrocytes wherein a sample of whole blood treated with an anticoagulant is brought into contact with a quantity of a multifunctional reagent for erythrocytes, sufficient to produce the lysis of at least 95% of the erythrocytes in less than 30 minutes or to provoke their sphering in order to allow their analysis by an automatic counting device.

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COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

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Invention Title:

Multifunctional Reagent for Erythrocytes

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

Multifunctional Reagent for Erythrocytes

The present invention relates to new reagents and methods for the treatment, and in particular the lysis of erythrocytes.

Red corpuscles are approximately one thousand times more numerous than leucocytes, they form a barrier to the analysis of the leucocytic fraction of the blood. Treatment of blood with a lysis reagent empties the red blood cells of their contents and makes it possible to isolate these cells from the analysis. An ideal lysis process comprises complete lysis of all the erythrocytic elements, without the slightest effect on the morphology and the viability of the leucocytes.

Hypotonic lyses are known among the numerous lysis reagents and methods. But a deformation of the leucocytes is observed.

Detergents are also known, but these produce a deterioration in the membrane of the leucocytes which are therefore counted by default.

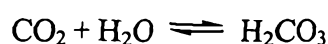
Small neutral, generally lipophilic molecules are also used such as alcohols or aldehydes but these have the drawback of being toxic for the leucocytes.

In fact, reagents containing amines have performance values closer to an ideal lysis process. The lysis reagents containing amines include the ammonium chloride reagent, the use of which is very widespread and the reagent based on nitrogenated heterocycles, as described in FR-A-2 778 413. Lysis reagents with ammonium chloride contain 155mM NH₄Cl, 10mM KHCO₃ and can sometimes also contain 0.1mM EDTA.

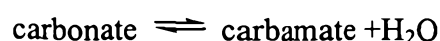
However, as described in FR-A-2 778 413, as the ammonium chloride lysis reagent has a rapid and complete lysis effect, it has a toxic effect on the leucocytes. The problem of the non-specific toxicity of ammonium chloride is probably linked to the presence of an ammonia concentration of approximately 1mM in the lysis mixture. The use of stronger bases, such as pyrrolidine and piperidine with a free base concentration of approximately 10μM, resolves the problem of non-specific toxicity, but gives a slower lysis reaction, this which is a drawback for its use in routine laboratories.

It would therefore be desirable to have available new reagents and lysis methods for erythrocytes with a more rapid and more complete action.

After much research the Applicant has surprisingly discovered that lysis reagents, and in particular reagents containing amines in the presence of a carbamate and/or of a catalyst of the reaction



and/or of the reaction

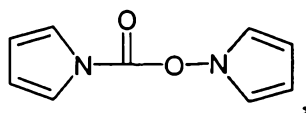


which are the two reactions involved in the formation of carbamate during the absorption of CO₂ by the erythrocyte, brought about a more rapid and more complete lysis of the erythrocytes than in the absence of these reagents.

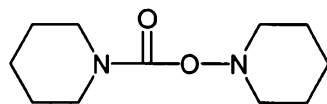
The influence of the presence of a carbamate in a lysis reagent on the speed of lysis is shown below in the experimental part.

According to a first aspect of the present invention there is provided a multifunctional reagent for lysing or sphering erythrocytes, said reagent comprising, in a quantity sufficient to produce lysis of erythrocytes or sphering of erythrocytes in such a way that they can be detected by a cytometer or an automatic counting device, a carbamate or an agent which induces formation of a carbamate by said erythrocytes from carbonate and a nitrogenated heterocycle or ammonium ions, during absorption of CO₂ by said erythrocytes.

According to a second aspect of the invention there is provided a carbamate chosen from pyrrolidine carbamate of formula



and piperidine carbamate of formula



According to a third aspect of the invention there is provided a process for lysing or sphering of erythrocytes wherein a sample of whole blood treated with an anticoagulant is brought into contact with a quantity of a reagent as defined in the first aspect of the invention, sufficient to produce the lysis of at least 95% of the erythrocytes in less than 30 minutes or to provoke their sphering in order to allow their analysis by an automatic counting device.

The present invention also provides lysed or sphered erythrocytes produced by the process of the third aspect of the invention.

According to a fourth aspect of the invention there is provided a process for sphering of erythrocytes wherein a sample of whole blood treated with an anticoagulant is placed in contact with a quantity of a reagent as defined in the first aspect of the invention, sufficient to provoke their sphering in order to allow their analysis by an automatic counting device.

The present invention also provides sphered erythrocytes produced by the process of the fourth aspect of the invention.

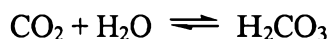
According to a fifth aspect of the invention there is provided a process for the preparation of leucocytes, said process comprising a stage of lysing erythrocytes using a multifunctional reagent as defined above.

The present invention also provides leucocytes prepared by the process of the fifth aspect of the invention.

The multifunctional reagent according to the invention permits either effective lysis of the erythrocytes or if desired the production of their simple sphering.

In preferential conditions for the implementation of the invention, the carbamate can be used in the molar concentration of 0.000001M to 0.1M, particularly 0.00001M to 0.01M and quite particularly 0.0001M to 0.005M. In wholly preferential conditions for the implementation of the lysis reagent described above, a concentration of 0.0004M is used.

In other preferential conditions for the implementation of the invention, the agent inducing the formation by the erythrocytes, from carbonate or an amine base, of a carbamate combined with the absorption of CO₂ by said erythrocytes, is a catalyst agent of the reaction



such as carbonic anhydrase, for example carbonic anhydrase I or carbonic anhydrase II. The enzyme can have varied isoelectric points and can have various sources as their origin. The activity of the enzyme is expressed in Wilbur-Anderson units. The carbonic anhydrase can be present in the concentration of 1 W-A U/litre to 1 000 000 W-A U/litre, particularly from 10 W-A U/litre to 500 000 W-A U/litre and very particularly from 100 W-A U/litre to 100 000 W-A U/litre. In wholly preferential conditions for the implementation of the lysis reagent described above, a concentration of approximately 50 000 W-A U/litre is used.

In the present application and in the following, the nitrogenated heterocycle in part constituting the carbamate can be for example bicyclic and preferably monocyclic. It can be unsaturated and in this case comprises for example 5, preferably 4, in particular 3, particularly 2 double bonds, and it is preferably saturated. It comprises for example from 3 to 8, in particular from 3 to 6 and particularly from 3 to 5, and very particularly 4 or 5 carbon atoms. It comprises 2, in particular 1 single nitrogen atom.

As saturated nitrogenated heterocycle there can for example be mentioned pyrazolidine, imidazolidine, the imidazoline and piperazine, in particular morpholine and particularly piperidine or pyrrolidine.

2b

In other preferential conditions for the implementation of the invention, the multifunctional reagent for erythrocytes moreover contains a nitrogenated heterocycle or an ammonium salt such as a halide like chloride.

The nitrogenated heterocycle can be one of those previously mentioned.

5 In a multifunctional reagent according to the invention, the nitrogenated heterocycle can be present in the molar concentration of 0.01 to 0.250M, particularly 0.08 to 0.19M and very particularly 0.12 to 0.18M. In wholly preferential conditions for the implementation of the multifunctional reagent described above, a concentration of 0.17M is used.

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The concentrations of the compound concerned in the reaction medium (multifunctional reagent + blood sample) during erythrocytic lysis are preferably 0.01 to 0.225M, particularly 0.072 to 0.17M and very particularly 0.11 to 0.17M.

In a multifunctional reagent according to the invention using carbonic anhydrase, the carbonate or hydrogen carbonate can be dispensed with as the blood serum naturally contains them. In yet other preferential conditions for the implementation of the invention, for example to accelerate lysis, the multifunctional reagent for erythrocytes moreover contains a carbonate or a hydrogen carbonate.

Sodium or potassium carbonate or hydrogen carbonate can for example be mentioned.

In a multifunctional reagent according to the invention, the carbonate or the hydrogen carbonate can be present up to the molar concentration of 0.1M, particularly up to 0.01M, and very particularly up to 0.005M. In very preferential conditions for the implementation of the multifunctional reagent described above, a concentration of 0.0025 M is used.

The concentrations of the compound concerned in the reaction medium (multifunctional reagent + blood sample) for erythrocytic lysis are preferably 0.0001 to 0.1M, particularly 0.001 to 0.01M and very particularly 0.0001 to 0.005M.

In yet other preferential conditions for the implementation of the invention, the multifunctional reagent for erythrocytes moreover contains a protective agent against the deterioration of the leucocytes such as a fixation agent, in particular an aliphatic aldehyde such as in C₁-C₅, for example paraformaldehyde and particularly formaldehyde.

The aliphatic aldehyde can be present in a concentration of 0.01% to 5%, particularly 0.14% to 1% and very particularly 0.1% to 0.5%.

In yet more preferential conditions for the implementation of the invention, the multifunctional reagent of the invention also comprises an effective quantity of an anticoagulant agent.

Heparin, in particular ion citrate, EGTA and particularly EDTA can for example be mentioned as anticoagulant agent.

The pH of the mixture without buffer at neutral pH during and after lysis tends to increase, thus provoking cell degradation. The use of a buffer of approximately neutral pH is therefore desirable. Despite the fact that the buffer tends to inhibit lysis, a small quantity of buffer (for example 1-20mM) can be used thanks to the accelerating effect of a catalyst such as carbonic anhydrase. The use of a carbamate according to the invention allows the use of a buffer of approximately neutral pH.

This is why, in other preferential conditions for the implementation of the invention, the multifunctional reagent of the invention also comprises an effective quantity of a buffer agent particularly pH6.5 to 7.5. There can for example be mentioned as buffer agent MES (2-(N-morpholino)ethane sulfonic acid), in particular MOPS (3-(N-morpholino)propane sulfonic acid) and particularly HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid)) and particularly the buffer DIPSO pH7.5.

In a multifunctional reagent according to the invention, the buffer agent can be present in the molar concentration of 0.0001 to 0.050M, particularly 0.0005 to 0.03M and very particularly 0.001 to 0.010M.

In preferential conditions for the creation of the multifunctional reagent described above without fixing agent, 0.17M of pyrrolidine hydrochloride, 2.5mM of potassium hydrogen carbonate, 3mM of DIPSO buffer pH7.5, 10mg/L of carbonic anhydrase and 0.1mM of EDTA are used.

In very preferential conditions for the creation of the multifunctional reagent described above with fixing agent, 0.3% of formaldehyde is also used.

As seen above, the catalyst agent of the reaction



or



such as carbonic anhydrase, induces the formation by the erythrocytes, from carbonate or from an amine base, of a carbamate combined with the absorption of CO_2 by said erythrocytes. The carbamates of the nitrogenated heterocycles can also be used directly to produce lysis of the erythrocytes. Some of these are new products.

This is why a subject of the present application is also a carbamate chosen from pyrrolidine carbamate, piperidine carbamate.

The reagents which are the subject of the present invention possess very advantageous properties. They are endowed in particular with remarkable properties which are lytic with regard to erythrocytes.

These properties are illustrated below in the experimental part. They justify the use of the reagents described above, in a method or a process for lysis or sphering of erythrocytes. Lysis of the red blood cells allows the analysis and/or triage using a flow cytometer of the leucocytes and their sub-populations, platelets, red blood cell residues after lysis, or any other cellular or suspended, element added or not, such as for example spherules.

They also justify the use of the reagents described above, in a process for the preparation of leucocytes.

A subject of the present application is also therefore a method of lysis of the erythrocytes in which a sample of whole blood treated with a anti-coagulant such as EDTA, EGTA, heparin or the citrate ions, is subjected to the action of a lysis reagent described above, to produce at least 95% lysis of the erythrocytes in less than 30 minutes.

It is possible to operate in the absence or in the presence of monoclonal antibodies in order to carry out the labelling of the leucocytic cells. These antibodies can be bound or not bound to a fluorescent compound such as those described below. In preferential conditions of implementation, these antibodies are bound to a fluorescent compound.

The multifunctional reagents described above can be used as follows:

A sample of 0.1mL of blood treated with an anti-coagulant, and incubated beforehand with a monoclonal antibody or a monoclonal antibody combined with a fluorescence marker, or a mixture of monoclonal antibodies combined with fluorescence markers is brought into contact with 2mL of multifunctional reagent above and left for ten minutes during which the lysis finishes. Such markers are for example CD45-FITC (CD45 combined with fluorescein isothiocyanate) or CD14 combined with phycoerythrin and are marketed for example by the companies Dako, Becton and Dickinson or

Beckman Coulter. A reading in a cytometer for example of Becton and Dickinson Facscan or Beckman Coulter XL type is then carried out either immediately, or up to 3 days after lysis.

In preferential conditions for the implementation of the method of lysis according to the invention, the preferential conditions described above for the multifunctional reagent are chosen.

The systems of lysis covered by the invention can also be used in the presence of a permeation agent such as an aliphatic alcohol or a detergent. A permeation reagent allowing an intracellular immunolabelling including a catalyst such as carbonic anhydrase or a carbamate is also part of the invention. Such a lysis and permeation stage can be carried out optionally after fixation by an aliphatic aldehyde.

In preferential conditions for the implementation of the process described above, a sample of whole blood is brought into contact with a quantity of a reagent above sufficient to produce the lysis of at least 95% of the erythrocytes in less than 10 minutes.

In other preferential conditions for the implementation of the process described above, the pH of the mixture of the reagent and the sample is between 4 and 9.

But the multifunctional reagent according to the invention also allows the production of the simple sphering of the erythrocytes.

This is why a subject of the present application is also a process of sphering (swelling) of the erythrocytes wherein a sample of whole blood treated with an anticoagulant is brought into contact with a quantity of a reagent above sufficient to provoke the sphering of the erythrocytes in such a way as to allow their analysis by an automatic counting device.

In preferential conditions for the sphering of the erythrocytes a sample of blood is brought into contact with a quantity of sphering reagent sufficient to produce sphering in 10 second to 400 second intervals. To avoid lysis of the erythrocytes in a time interval and to control the speed of the sphering, the multifunctional reagent according to the invention can be diluted in a preferably isotonic solution. Between 0 and 10 times the same volume of diluent, preferably between 0.5 and 5 times and particularly between 1 and 3 times can for example be added. In particularly preferential conditions, the choice of isotonic solution is IsoFlow[®] from Beckman Coulter which contains NaCl: 7.92g/L, KCl: 0.4g/L, NaH₂PO₄: 0.19g/L, Na₂HPO₄: 1.95g/L, EDTA: 0.38g/L, 2-phenoxyethanol: 3g/L, and NaF: 0.3g/L.

In other preferential conditions, one or more adjuvants such as fixation reagents, colorants of nucleic acids, and/or isosphering agents such as maltoside (D.H.Tycko, US-A-5 194 909) are added to the sphering reagent.

As has been seen above, the multifunctional reagent of the invention is not aggressive for the leucocytes and can therefore be used with a view to their preparation. The lysis systems covered by the invention are therefore also tools for the preparation of viable leucocytic cells and can replace the technique of cell separation on Ficoll-Hypaque. The preparation of the viable cells after lysis of blood, pathological blood, bone marrow and any other body fluid, has an application for example for the purposes of research for functional cellular tests, freezing and storage of the cells, etc. As far as the control of cellular viability is concerned, the lysis methods can be used with viability colorants such as eosin, blue trypan, 7-AAD, LDS 751 etc. Finally, as the lysis according to the studies of the Applicant

is a function of the capacity of the erythrocytes to absorb CO₂, the system of lysis can be used as a functional parameter of the erythrocytes.

This is why a subject of the present application is also a process for the preparation of leucocytes wherein it comprises a stage of lysis of the erythrocytes using a multifunctional reagent such as described above.

The preferential conditions for the implementation of the processes described above also apply to the other subjects of the invention referred to above

Figure 1 represents a size-structure diffusion diagram of a blood sample analysed using flow cytometry (Beckman Coulter XL) after lysis according to example 1. Region A corresponds to the lymphocytes, region B corresponds to the monocytes and region C to the granulocytes.

Figure 2 represents a size-structure diffusion diagram of a blood sample analysed using flow cytometry (Beckman Coulter XL), after spherizing according to Example 6. After contact of the erythrocytes with the spherizing agent, described in Example 6, the size-structure occurrences of the erythrocytes are represented in 4 intervals of 100s. (figure 2A). The spherizing and the homogenisation of the erythrocytes which results from this over time are illustrated in the size-structure diagrams; figure 2B, after 100s; figure 2C, after 200s; figure 2D, after 300s; figure 2E, after 400s.

The following examples illustrate the present application.

Example 1:

A multifunctional reagent for erythrocytes having the following composition was prepared:

Pyrrolidine chloride	170mM
Potassium hydrogen carbonate	2.5mM
DIPSO buffer pH 7.5	3mM
Bovine carbonic anhydrase (SIGMA)	10mg/L
(50000 W-A units)	
EDTA	0.1mM
pH 7.3	

Example 2:

A multifunctional reagent for erythrocytes having the following composition was prepared:

Pyrrolidine chloride	170mM
Potassium hydrogen carbonate	2.5mM
DIPSO buffer pH 7.5	3mM
Bovine carbonic anhydrase (SIGMA)	10mg/L
(50 000 W-A units)	
EDTA	0.1mM
Formaldehyde	0.1%
pH 7.3	

Example 3:

A multifunctional reagent for erythrocytes having the following composition is prepared:

Piperidine chloride	170mM
Potassium hydrogen carbonate	2.5mM
DIPSO buffer pH 7.5	3mM
Bovine carbonic anhydrase (SIGMA)	10mg/L
(50 000 W-A units)	
EDTA	0.1mM
pH 7.3	

Example 4:

A multifunctional reagent for erythrocytes having the following composition was prepared:

Ammonium chloride	155mM
Potassium hydrogen carbonate	2.5mM
DIPSO buffer pH 7.5	3mM
Bovine carbonic anhydrase (SIGMA)	10mg/L
(50 000 W-A units)	
EDTA	0.1mM
pH 7.3	

Example 5: performance of a lysis

100 μ L of whole blood is mixed with 1mL of a lysis agent of Example 1. The solution is left to rest at ambient temperature for 10 minutes and the leucocytes are counted by cytometry.

Examples 6 to 8: Preparation of pyrrolidine, piperidine and morpholine carbamates

A mixture of sodium bicarbonate and a quantity of each compound (molar ratio 1:2), was heated to 100°C accompanied by stirring. The reaction is characterised by a solidification of the mixture, which occurs after approximately an hour. The sought carbamates were recovered after extraction with methanol. The molecular structure of these synthesised compounds was verified by NMR spectrometry ^{13}C at 200MHz.

These analyses (in deuterated methanol) made it possible to obtain the following chemical shifts (δ : ppm):

1/ Pyrrolidine carbamate: 26.44; 27.24; 47.32; 47.63 (CH_2 ; 165.02 ($\text{C}=\text{O}$))

2/ Piperidine carbamate: 25.69; 26.58; 27.39; 28.71; 47.65 (CH_2 ; 162.76 ($\text{C}=\text{O}$))

3/ Morpholine carbamate: 47.14; 68.42 (CH_2 ; 162.81 ($\text{C}=\text{O}$))

Example 9: Preparation of a sphering agent

A sphering agent was prepared by mixing 2 volumes of reagent according to Example 1 and a volume of Isoflow[®], an isotonic composition marketed by BECKMAN COULTER.

Example 10: Cytometry after sphering

A volume of blood of 4 μ L was placed in contact with 1mL of the sphering agent of Example 9. The process of sphering was tracked by cytometry as a function of time in a Beckman Coulter XL cytometer. The results are presented in figure 2 mentioned above.

Experiment 1:

The speed of lysis by the ammonium ion and by nitrogenated heterocycles in the presence of KHCO_3 (10mM) or in the presence of their respective carbamate derivatives (0.4mM) was studied. A volume of 2mL of reagent was used for a volume of 0.1mL of whole blood treated with anticoagulant (EDTA). Variations in pH were obtained by adding a 1M aqueous solution of HCl before the addition of carbamate. The carbamates (100mM in methanol) were added to the mixtures at the start of the reaction. The pH of the reaction mixture was measured. The lysis process was tracked spectrophotometrically (measurement of optical density at 700nm). The lysis time is determined by the time necessary to obtain a minimum level.

The results obtained are the following:

	Time necessary to reach total lysis (maximum transparency)	pH during the lysis reaction
NH ₄ Cl (150mM), KHCO ₃ (10mM)	7min	7.40
Idem	4min	7.03
NH ₄ Cl (150mM) ammonium carbamate (0.4mM)	1min 50	7.20
Pyrrolidine (150mM), KHCO ₃ (10mM)	7min	7.20
Idem	6min	7.75
Pyrrolidine, pyrrolidine carbamate (0.4mM)	3min	7.66
Piperidine (150mM), KHCO ₃	15min	7.30
Idem	10min	7.8
Piperidine (150mM), piperidine carbamate (0.4mM)	6min 40	7.68
Morpholine (150mM), KHCO ₃ (10mM)	12min	6.82
Morpholine (150mM), morpholine carbamate (0.4mM)	8min	6.90

Conclusion: It will be seen that the addition of the different carbamates to the ammonium ion and to the nitrogenated heterocycles induces a much greater speed of lysis than that obtained by adding hydrogen carbonate at a concentration 25 times that of the carbamate. As the table shows, the lytic effect of the carbamates does not depend on the pH.

5 **Experiment 2. Demonstration of the catalyst role of carbonic anhydrase in the reaction carbonate ↔ carbamate + H₂O;**

The reaction $(\text{NH}_4)_2\text{CO}_3 \leftrightarrow \text{NH}_2\text{CO}_2\text{NH}_4 + \text{H}_2\text{O}$ was studied through the conversion of the ammonium carbamate into ammonium carbonate measured by their difference in solubility in an aqueous solution of 85% of acetone in which the carbonate precipitates and not the carbamate.

Reaction mixture	Duration before complete precipitation in 85% of acetone
100mM NH ₂ CO ₂ NH ₄	240 min
100mM NH ₂ CO ₂ NH ₄ + 0.001mg/mL of carbonic anhydrase	10 min

10 **Conclusion:** The results show that the carbonic anhydrase, in addition to catalysing the conversion $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3$ as is well known, also catalyses the conversion carbonate ↔ carbamate + H₂O

Experiment 3: Demonstration of the effect on the speed of lysis of carbonic anhydrases originating from different sources.

15 In a lysis reagent according to Example 1, the component carbonic anhydrase at 50 000 W-A U/L was replaced by preparations of carbonic anhydrase as indicated in the table, each at 5000W-A U/L. The lysis process was tracked spectrophotometrically (measurement of the OD at 700nm). The duration of the lysis is determined by the time necessary to achieve a minimum level.

Supplier	Reference	Origin	Speed of lysis at 5000W-A U/L
Control	without enzyme		15.00min
Sigma	C-3934	Bovine	7.30min
Biozyme	CABI	Bovine	7.06min
Biozyme	CABII	Bovine	8.30min
Sigma	CAII, C-2522	Bovine	8.51min
Sigma	CAII, C-6165	Human	7.06min
Sigma	CAI, C-4396	Human	5.06min
Sigma	CAI, C-5290	Human	3.24min
Sigma	CAI, C-1266	Rabbit	8.00min

Conclusion. The different preparations of carbonic anhydrase all increase the speed of lysis.

The claims defining the invention are as follows:

1. A multifunctional reagent when used for lysing or sphering erythrocytes, said reagent comprising, in a quantity sufficient to produce lysis of erythrocytes or sphering of erythrocytes in such a way that they can be detected by a cytometer or an automatic counting device, a carbamate or an agent which induces formation of a carbamate by said erythrocytes from carbonate and a nitrogenated heterocycle or ammonium ions, during absorption of CO₂ by said erythrocytes.

2. A multifunctional reagent when used for lysing or sphering erythrocytes according to claim 1, wherein said agent is a catalyst agent of the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$.

3. A multifunctional reagent for erythrocytes according to claim 2, wherein the catalyst agent is carbonic anhydrase.

4. A multifunctional reagent for erythrocytes according to any one of claims 1 to 3, wherein said reagent also contains a nitrogenated heterocycle or an ammonium salt.

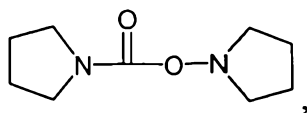
5. A multifunctional reagent for erythrocytes according to any one of claims 1 to 4, wherein said reagent also contains a carbonate or a hydrogen carbonate.

6. A multifunctional reagent for erythrocytes according to any one of claims 1 to 5, wherein said reagent also contains an agent which protects against deterioration of leucocytes.

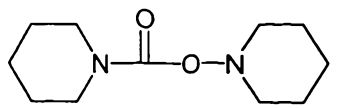
7. A multifunctional reagent for erythrocytes according to claim 6, wherein the agent which protects against deterioration of leucocytes is an aliphatic aldehyde.

8. A multifunctional reagent for erythrocytes, said reagent being substantially as hereinbefore described with reference to any one of the examples.

9. A carbamate chosen from pyrrolidine carbamate of formula



and piperidine carbamate of formula



10. A carbamate chosen from pyrrolidine carbamate and piperidine carbamate, obtainable by a process substantially as hereinbefore described with reference to Examples 6 to 8.

11. A process for lysing or sphering of erythrocytes wherein a sample of whole blood treated with an anticoagulant is brought into contact with a quantity of a reagent as defined in any one of claims 1 to 8, sufficient to produce the lysis of at least 95% of the erythrocytes in less than 30 minutes or to provoke their sphering in order to allow their analysis by an automatic counting device.

12. A process according to claim 11, wherein a sample of whole blood is placed in contact with a quantity of reagent sufficient to produce the lysis of at least 95% of the erythrocytes in less than 30 minutes.

13. A process according to claim 11 or claim 12, wherein the pH of the mixture of the reagent and of the sample is between 4 and 9.

14. A process for lysing or sphering of erythrocytes, said process being substantially as hereinbefore described with reference to any one of Examples 1 to 5 or 10.

15. A process for sphering of erythrocytes wherein a sample of whole blood treated with an anticoagulant is placed in contact with a quantity of a reagent as defined in one of claims 1 to 8, sufficient to provoke their sphering in order to allow their analysis by an automatic counting device.

16. A process for sphering of erythrocytes, said process being substantially as hereinbefore described with reference to any one of Examples 1 to 4, 9 or 10.

17. Lysed or sphered erythrocytes produced by the process of any one of claims 11 to 14.

18. Sphered erythrocytes produced by the process of claim 15 or 16.

19. A process for the preparation of leucocytes, said process comprising a stage of lysing erythrocytes using a multifunctional reagent as defined in any one of claims 1 to 8.

20. A process for the preparation of leucocytes comprising a stage of lysing erythrocytes using a multifunctional reagent, substantially as hereinbefore described with reference to any one of the examples.

21. Leucocytes prepared by the process of claim 19 or claim 20.

Dated 19 November, 2002

Immunotech

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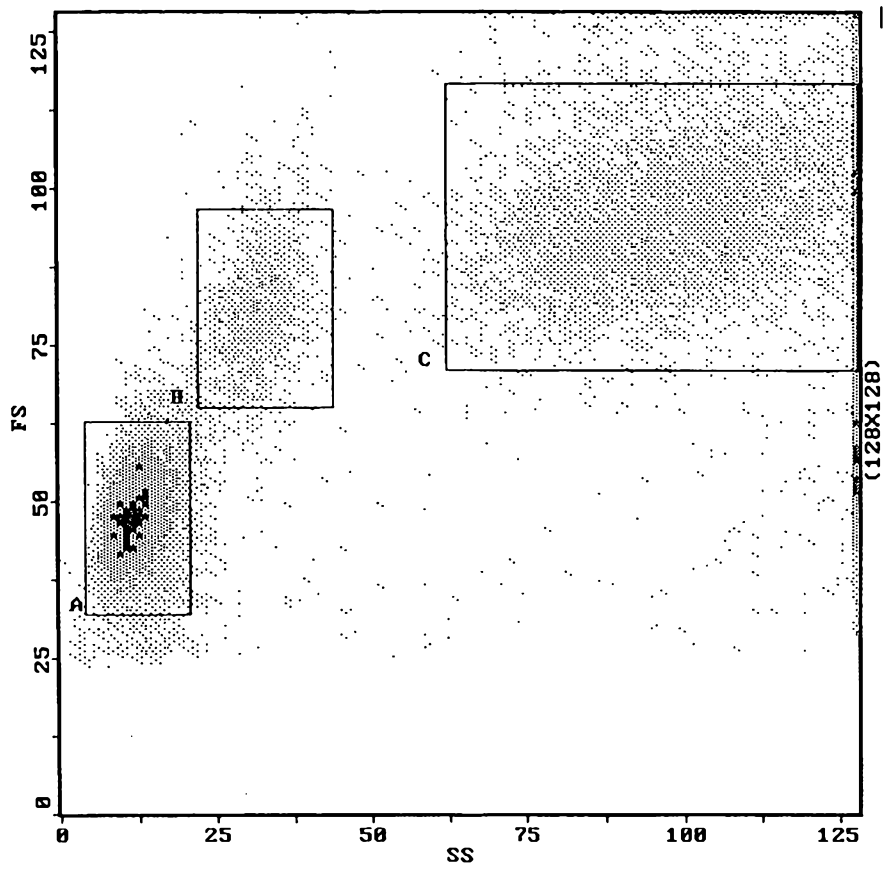


Figure 1

