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Stability and functional properties of haemoglobin freeze-dried in the presence of four protective substances after prolonged storage: dose-effect relationships

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Freeze-dried haemoglobin samples protected during the desiccation by sucrose, arginine aspartate, lysine aspartate, sodium-zinc EDTA and Ficoll 70 have been stored under air at 4 °C for 15 months. The analysis showed that sufficient concentrations of sucrose and of the amino-acid salts prevent the oxidation of haemoglobin and maintain its functional properties. Relationships between the concentrations of these compounds and the methaemoglobin levels before and after storage were calculated. They define theoretical concentration points where methaemoglobin would not be found after storage. Sucrose is slightly more effective than the amino-acid, but oppositely, EDTA and Ficoll 70 do not allow prolonged storage of freeze-dried haemoglobin.

Freeze-drying haemoglobin without denaturing it was made possible by the demonstration that many simple chemicals such as carbohydrates (Smith & Pennell 1952; Labrude et al 1976 b, 1980 b), amine buffers (Labrude & Vigneron 1980 a), amino acids (Labrude et al 1980 c) EDTA salts (Vigneron & Labrude 1980) and two macromolecules (Ficoll 70 and Ficoll 400) (Labrude et al 1981 a) have properties that protect against the effects of freeze-drying.

Few studies on the storage of the freeze-dried materials have been reported so far, and these were for limited times—4 months (Greenburg et al 1975) to one year (Pristoupil et al 1980)—or were concerned with only a few protective additives such as glucose or sucrose (De Venuto et al 1979). Simultaneous testing of several protectors, each one used at several concentrations and for a prolonged period of storage, ought to make it possible to demonstrate which compounds are most effective and to define the minimum concentration of each at which haemoglobin suffers little or no denaturation during the period in question. We report here the results of a study of the storage of freeze-dried haemoglobin at 4° C for 15 months in the presence of various concentrations of protectors belonging to four different chemical classes, of which three had not, to our knowledge, been studied in these conditions—sucrose, arginine aspartate, lysine aspartate, sodium zinc EDTA, and Ficoll 70. The experimental values obtained were used to calculate correlations between the doses of the protector and the functional state of the haemoglobin after storage, and to compare the effectiveness of the two best compounds.

MATERIALS AND METHODS

The haemoglobin solution was prepared as described before (Labrude et al 1981 a, b) and adjusted to 40 g litre^{-1} (2·5 mM in haemoglobin monomer). The protectors—sucrose (Merck), L-arginine L-aspartate and L-lysine L-aspartate (Sigma), sodium zinc EDTA (Prolabo), and Ficoll 70 (Sigma)—were dissolved at the concentrations indicated in Tables 1–3; the lowest concentrations yielded levels of methaemoglobin not exceeding 10% immediately after freeze-drying, and the highest concentrations were those that we used in earlier work (Labrude et al 1981 a, b).

The freeze-drying tests and the storage were carried out as described before (Labrude et al 1981 b), in series each containing one control. After 15 months, each freeze-dried sample was examined for appearance and colour, and was reconstituted by the addition of 5 ml of Sörensen 66 mM phosphate buffer, pH 7·8, and immediately analysed by the tests described before (Labrude et al 1981 b), as follows: estimation of methaemoglobin (Evelyn & Malloy 1938), measurement of the oxygen saturation, measurement of the pH, recording of the visible spectrum and calculation of the ratio of the optical densities at 576 and 560 nm, and plotting of the

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haemoglobin dissociation curve with determination of the P50 and calculation of the Hill's coefficient.

RESULTS

On removal from the freeze-drier, the unprotected freeze-dried materials were brown and contained 49 ± 10% of methaemoglobin (Labrude et al 1981 a); after 15 months of storage, they dissolved slowly but completely, yielding dark solutions that contained almost 100% methaemoglobin.

After storage, all the materials dried in the presence of a protector had, when dry, a colour ranging from brown to red with decreasing methaemoglobin levels. They always dissolved completely —the less methaemoglobin, the more rapidly they dissolved—but centrifugation was necessary for the least-well-preserved materials, which were turbid after dissolution. Dichroism was often observed with the low and medium levels of methaemoglobin: the solutions were redder than the corresponding dried materials.

Since the oxyhaemoglobin saturation was approximately in accord with the methaemoglobin levels, we report only the methaemoglobin value in the Tables.

Sucrose

The results obtained in the three series of tests carried out with sucrose are shown in Table 1. At time zero, the methaemoglobin levels obtained decreased significantly with increasing molarity of the protector (n = 18, r = −0·819, P <0·001); this was also true after storage (n = 18, r = −0·840, P <0·001). For sucrose concentrations greater than 0·12 M the methaemoglobin level was less than 10%,

Table 1. Functional properties of the three series of freeze-dried haemoglobin samples prepared in the presence of sucrose after 15 months of storage at 4 °C.

		Met Hb %					Spectra
Series No.	Concn (M)	Before storage	After storage	pH	p50 (kPa)	Hill's number	OD 576 / OD 560
2	0·035	8	50·8	7·25			
1	0·043	10	58·2	7·31			
2		6	42·2	7·23			
3	0·049	7·4	32·2	7·23			
1	0·058	3·4	47·4	7·28			
2		3·7	25·7	7·23			
3	0·064	3·3	25·5	7·21			
1	0·073	4·2	27	7·30			
2		2·6	12·9	7·20	3·32	2·66	1·61
3	0·079	1·7	9·8	7·20	3·52	2·50	1·62
1	0·087	1·8	16·3	7·25	2·92	2·52	1·66
1		2·7	18·1	7·25	2·79	2·77	1·65
2	0·093	2·5	7·1	7·20	3·25	2·56	1·69
1	0·102	2·1	13·8	7·28	2·79	2·78	1·61
3		3·2	6·5	7·20	2·99	2·50	1·67
1	0·116	1·3	19·6	7·23	2·86	2·45	1·65
2		1	4	7·20	3·45	2·76	1·72
3	0·131	2	9·6	7·23	3·19	2·80	1·63

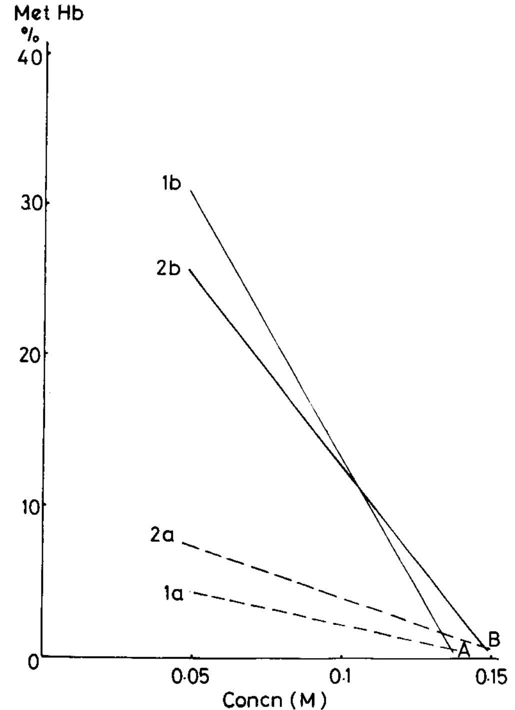

FIG. 1. Relationships between methaemoglobin percent and protector concentrations before (- - - -) and after storage (——) for sucrose (1a and 1b) and amino-acid salt (2a and 2b). A and B represent theoretical concentrations for which methaemoglobin would be non-existent after storage.

which was confirmed by plotting the regression line (Fig. 1).

This satisfactory preservation was confirmed by the high oxyhaemoglobin values, by the absorbance ratios in the region of the haem absorption on spectrophotometry, and particularly by the maintenance of the cooperativity demonstrated by the normal values of the Hill's coefficient. We looked to see if the increase in the methaemoglobin levels expressed by a coefficient R, equal to the percentage of methaemoglobin after storage divided by the percentage before, was inversely proportional to the sucrose concentration. In our experiment, it was not (n = 1[illegible], r= −0·342, N.S.).

The change of the Hill's coefficient was also not correlated with the methaemoglobin level (n = 10, r = −0·135, N.S.).

Amino acid salts

Owing to the great similarity between the effects of arginine aspartate and lysine aspartate (Labrude et al 1981 b), the values we found with a series of

Table 2. Functional properties of freeze-dried haemoglobin samples containing one amino-acid salt after 15 months of storage at 4 °C.

Series No.	Amino-acid salt	Concn (M)	Met Hb (%) Before storage	Met Hb (%) After storage	pH	p50 (kPa)	Hill's number	Spectra OD 576 / OD 560
1	Arg. Asp.	0·05	8·5	30·3	7·08			
1	Arg. Asp.	0·07	5·9	18·4	7·04			
1	Arg. Asp.	0·09	2·3	11·0	7·00			
2	Lys. Asp.		6·2	14·4	7·22	2·99	2·63	1·67
1	Arg. Asp.	0·12	1·8	5·5	7·07	3·19	2·79	1·68
2	Lys. Asp.		3·2	8·3	7·14	2·92	2·69	1·70
1	Arg. Asp.	0·14	1·8	6·6	6·96	2·99	2·79	1·70
2	Lys. Asp.	0·15	3·8	11·6	7·08	2·86	2·74	1·65
2	Lys. Asp.	0·16	3·0	5·1	7·10	3·19	2·88	1·74
2	Lys. Asp.	0·18	2·7	7·0	7·18	2·79	2·59	1·74
2	Lys. Asp.	0·20	3·2	4·4	7·05	3·19	2·50	1·70

samples of each salt were considered together, as if they had come from a single experiment (Table 2).

The methaemoglobin levels before storage, which were always low, were to some extent inversely correlated with the concentration of the protector; however, the correlation coefficient was quite low (n = 11, r = −0·621, P <0·05), showing that these salts were very effective even at low concentrations and that increasing the doses improved the quality of the measured results little or not at all.

The lowest concentration of protector gave a high level or methaemoglobin after storage, but its values declined very rapidly.

Curves of the percentage of methaemoglobin as a function of the molarity of the protector can be converted into straight lines by logarithmic transformation, as we have already shown (Labrude et al 1981 a). The correlation was satisfactory in both methods of representation, and had the values r= −0·788 and r= −0·825 respectively, with P<0·01 and n = 11. The excellent stability of almost all the samples was confirmed by the spectrophotometric results and the Barcroft's curve. As with sucrose, we found no correlation between the molarity of the amino acid and an increase in the oxidation of the haemoglobin (n = 11, r= −0·501, N.S.), nor between the amount of methaemoglobin and the Hill's coefficient (n = 8, r− −0·098, N.S.).

We compared the effectiveness of sucrose and of arginine aspartate before and after storage, considering only the values obtained for the same molarities, that is, between 49 and 140 mM. For sucrose at the initial time, there was a dose-effect relation—y= −45·73 × +6·74 (n = 15, r= −0·692, P<0·01)—as also for the amino acid salt—y= −68·83 × + 10·88 (N = 7, r= −0·864, P<0·02). The straight-line plots (Fig. 1) show that sucrose was the more effective of the two. Comparison of these two regression lines by the Fisher-Snedecor identity test shows that they are separate.

After 15 months of storage, the regression lines were determined:

y = −342·1 × +47·43 with n = 15, r = 0·695, P<0·01 for sucrose

y= −249·5 × + 37·7 with n = 7, r= −0·928, and P<0·01 for arginine aspartate.

These two straight lines were still separate by the previous test; but it is particularly important to note that they cross near 0·1 M and that below this molarity the amino acid was the more effective.

Extending the straight lines for the two compounds (Fig. 1), defines at their convergence two points, A and B, that correspond in theory to the concentration of protector that needs to be added to 10 ml of a 40 g litre^{-1} haemoglobin solution in order that the methaemoglobin level, which starts out at less than 2% immediately after drying, is to remain at that value after 15 months of storage at 4 °C; here again sucrose turned out to be more effective than the amino acid salt, since point A occurs at a lower concentration and a lower methaemoglobin level than with arginine aspartate (point B).

In fact, although these two compounds are not identical in effectiveness, the difference is more theoretical than practical, since both give very satisfactory results at moderate, comparable concentrations.

EDTA

The results obtained with EDTA are shown in Table 3. At no concentration did it preserve haemoglobin

Table 3. Functional properties of freeze-dried haemoglobin samples obtained with Na_2 Zn EDTA salt after 15 months of storage at 4 °C.

Series No.	Concn (M)	Met Hb (%) Before storage	After storage	pH
1	0·05	9·3	50·9	7·05
2	0·06	8·2	52·7	7·15
2	0·08	8·5	41·9	7·15
1	0·09	10·0	41·5	7·04
2	0·10	6·2	41·7	7·14
1	0·12	8·2	34·2	7·05
2	0·14	6·8	24·0	7·10
1	0·16	6·2	28·9	7·00
2	0·18	6·4	28·1	7·09
1	0·20	5·3	26·8	6·98

satisfactorily. We found no correlation between the level of methaemoglobin and the concentration of protector before and after preservation, nor between the ratio expressing the increase in methaemoglobin and the molarity ($n = 10$, $r = 0{\cdot}418$, N.S.).

Ficoll 70

Ficoll 70 also was not a good preservative. For concentrations of this material between 700 and 400 μM, the level of methaemoglobin was respectively from 3 to 10% before storage and from 35 to 56% after 15 months.

DISCUSSION AND CONCLUSION

This study presents the results for the storage of haemoglobin for 15 months at 4 °C after it had been freeze-dried in the presence of one of four protectors. A comparable storage period (16 months) has, so far as we know, been described only by Bonderman et al (1980).

In our study, sucrose gave satisfactory results at concentrations of 120 mM and above, and the cooperativity of the haemoglobin was very well preserved. Therefore sucrose belongs to the class of protectors against the effects of freeze-drying that are effective over a long period. This point has not been reported before, since previous studies have been only for shorter periods. However, the correlation figures show that this result was achieved in our experimental conditions only when the concentration was much higher than the lowest that yielded good results at time zero. A concentration of 50 mM was more than enough in the case of a freeze-dried material intended for immediate use, whereas the concentration had to be tripled for prolonged storage at 4 °C.

Sucrose was also included in the study of Bonderman et al (1980), who found that mixed with Ficoll 400 it gave results that were unsatisfactory even after only one year at 5 °C. It would be interesting to know why. We can, from our experimental results, offer two possible explanations. The first is that the concentration of sucrose (290 mM) may have been too high, since we obtained similar results after 15 months for concentrations between 150 and 250 mM (unpublished results). It is known (Simatos et al 1974) that various sugars, including sucrose, impede thorough desiccation and therefore give the dried materials a residual humidity, which presumably increases with increasing concentrations of the carbohydrate. This would certainly hamper the stability of haemoglobin. Estimations of the residual humidity might yield useful information. The second hypothesis that we could offer concerns the possibly harmful presence of Ficoll 400 'alongside' sucrose in the work of Bonderman et al (1980). Our results show that Ficoll 70 was not effective for long, although it worked almost as well as its higher homologue during the drying (Labrude et al 1981 a), which suggests that freeze-dried haemoglobin samples containing only Ficoll 400 would not keep well either.

Amino acid salts seem not to have been studied hitherto in the freeze-drying of haemoglobin (Labrude et al 1980 c). The present results confirm those presented recently (Labrude et al 1981 b) and show that these substances behave like sucrose: their effectiveness during storage was a function of their concentrations.

A concentration of 150 mM gave a low level of methaemoglobin after 15 months. Sodium zinc EDTA, which was one of the best salts of this acid in our hands (work in progress), did not preserve freeze-dried haemoglobin during storage, although the functional integrity of the haemoglobin immediately after the drying was as satisfactory as with other protectors. The same was true for Ficoll 70.

So of the various known classes of protective substances for the freeze-drying of haemoglobin, of which at least one representative has been the subject of a study of storage in real time, in our laboratory (Labrude 1976 a) or elsewhere (Pristoupil et al 1980), only sucrose and the amino acid salts give satisfactory and comparable results. These substances are therefore the types that should be studied more thoroughly: first, freeze-drying conditions should be chosen similar to those used in actual practice, as has already been attempted by De Venuto et al (1979) with glucose, though with a shorter storage period; then the samples should be

stored for 2 years at 4 °C; finally, if the results then obtained are satisfactory, the capacity of reconstituted solutions of haemoglobin on transfusion should be studied in animals.

With this aim, we have just performed nine tests of freeze-drying of 50- to 180-ml samples of haemoglobin solutions, frozen in the form of centrifuge pellets or of shells, protected with sucrose, lysine aspartate, or arginine aspartate at concentrations between 90 and 270 mM. The results recorded immediately after drying were very satisfactory—less than 5% methaemoglobin and a Hill's coefficient between 2·7 and 3·2.

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