The Reaction beetween Proteins and Reducing Sugars in the 'Dry' State

DRIED HUMAN BLOOD PLASMA

By C. H. LEA and R. S. HANNAN
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research
and R. I. N. GREAVES
Department of Pathology, University of Cambridge

(*Received* 5 *June* 1950)

In previous work (Lea & Hannan, 1949, 1950*a*, *b*) it has been shown that a pronounced interaction takes place between casein and glucose when a freeze-dried mixture is held at moderate temperatures and humidities, the predominant reaction in the early stages being a combination of free amino groups of the protein each with the reducing group of a sugar molecule. By this means as much as 7 % or more by weight of glucose can be attached to casein by a stable chemical link without the production of any obvious changes in the physical properties of the protein. Subsequently, marked discoloration, loss of solubility and other symptoms of deterioration develop, further quantities of sugar and other reactive amino-acid residues becoming involved at this stage.

The present investigation was undertaken because the plasma used for the preparation of dried plasma for human transfusions in this country is prepared from blood which has been taken into a preserving solution containing a large amount of glucose. If the same reaction occurs between dry plasma proteins and glucose, as has been observed between casein and glucose, the plasma proteins might be markedly altered and converted to virtually new proteins before any outward sign of deterioration became apparent. Clearly, such an alteration might be highly undesirable in a material injected in large quantities into humans.

The experiments outlined below indicate the extent to which changes of the type described can occur in dried blood plasma during preparation and storage. The possibility of similar changes must always be borne in mind whenever biological material containing reducing sugars is dried (Lea & Hannan, 1950*c*).

EXPERIMENTAL

The techniques used were mainly as previously described except that to provide a closer approach to practical conditions the main experiment was carried out at constant water content instead of at constant relative humidity. A few samples, however, were stored at constant relative humidity for purpose of comparison.

Human blood (420 ml.) from each of two donors was collected in the normal manner into 120 ml. of anticoagulant mixture consisting of 100 ml. of 2% disodium citrate and 20 ml. of 15% (w/v) glucose, the red cells were removed by centrifugation, the samples mixed, ampouled in 5 ml. portions and freeze dried by the centrifugal technique (Greaves, 1946); the condenser temperature was $-45°$, the drying temperature $-25°$, and the dried material was eventually allowed to rise to $+25°$. A further set of samples was prepared in a similar way, except that the blood was collected into disodium citrate without the addition of glucose.

The dried materials were further dried to a very low and uniform moisture content by standing *in vacuo* over P_2O_5 for 3 weeks at a temperature of 12·5°, and various batches were then allowed to absorb moisture from the atmosphere to a number of preselected water contents. The small amount of water remaining after the P_2O_5 drying was determined by taking samples to constant weight *in vacuo* at 37° over $Mg(ClO_4)_2$, this being taken as the definition of zero moisture content since higher temperatures involve the danger of loss of weight owing to the onset of the protein-sugar reaction. In this way samples were prepared with water contents of 0·3, 1·1, 1·9, 3·7, 5·2, 8·2 and 22·6% with added glucose, and 0·3, 1·1, 2·0, 3·7, 5·4, 7·9 and 22·1% without added glucose, all percentages being quoted as g. water/100 g. dry non-glucose solids for ease of comparison between the two batches. A further group of samples with zero moisture content (by definition) was prepared by drying *in vacuo* at 37° for 3 weeks over $Mg(ClO_4)_2$. When all the samples had attained the desired moisture contents the ampoules were filled with N_2, sealed and stored at 37 or 20°. The dry samples were sealed in vessels containing $Mg(ClO_4)_2$.

The water relations of both preparations were determined by allowing samples of 600 mg. of the material with 0·3% moisture to equilibrate at 37° to constant weight at various relative humidities. Samples of human blood serum dried with and without the addition of disodium citrate (1 g./150 ml.) were examined similarly.

The figures quoted for free amino-N values are based on the Van Slyke figures (30 min. reaction time) on the reconstituted plasma, corrected for the small figure attributable to the urea present. The urea content was about 20 mg./100 ml. plasma in both sets of samples, and did not change on storage as determined by manometric determination of the CO_2 produced after the action of urease. The free amino-N value of

plasma is due mainly to protein groups, since about 85% of the total could be removed by precipitation with trichloroacetic acid.

The glucose contents of the original undried plasma were determined manometrically using the specific oxidase notatin.

RESULTS

Samples containing added glucose

This plasma before drying had an initial content of 76·9 mg. amino-N/g. total N, and contained 696 mg. glucose/100 ml., corresponding to 0·78 of an equivalent of the amino-N. The pH of the reconstituted dried material was 7·9. Fig. 1 shows the effects

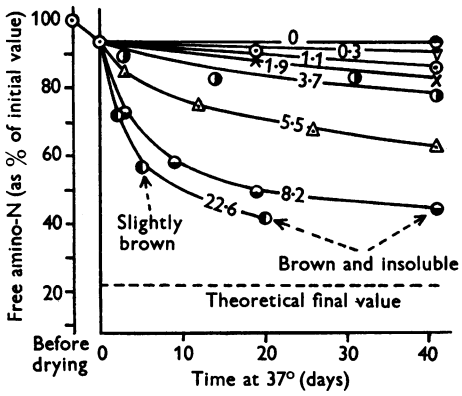

Fig. 1. The loss of free amino-N in dried human blood plasma containing added citrate and glucose on storage at 37° in presence of the indicated amounts of water (as g./100 g. dry non-glucose solids).

observed on storage at 37°, all amino-N figures being expressed as percentage of the initial figure. The theoretical loss corresponding to a one to one reaction of amino groups with all the available glucose is also indicated. From these results it can be seen that (*a*) the free amino-N content of the plasma decreases during storage, the rate being most rapid at first and falling off markedly with time. The initial rate and the extent of the reaction before the rate falls off are both increased by increase of water content. (*b*) The rate in the complete absence of water is negligible, a fact which had not been demonstrated clearly with the casein-glucose system. The present technique, however, is an improvement on that previously employed in that the preliminary drying was carried out at slightly higher temperature, storage was commenced at 37° *in vacuo* to remove the last traces of water as rapidly as possible, and the samples were subsequently sealed in individual vessels over a desiccant. (*c*) The reaction can proceed to an appreciable extent even during drying. It should be noted that this change was observed following freeze drying which was completed in 24 hr., the final temperature being 25°. Much greater change might have been expected if a final drying temperature of 80° had been used as advocated by some American workers for material without added glucose (Strumia & McGraw, 1943). (*d*) Physical evidence of deterioration is only apparent after considerable chemical reaction has taken place. The samples containing 8·2% of moisture lost about half of their total amino-N before the material was obviously brown and failed to dissolve rapidly. At lower moisture contents no marked browning was evident in 40 days at 37° although considerable interaction between protein and sugar had taken place.

Temperature coefficient. Assuming the temperature coefficient to be as high as for the reaction between casein and glucose (i.e. $Q_{10}^{20-30°} = 5·1$) the rates at 20° should be about one-eighteenth of those at 37°. A few samples were stored at 20° to test this relationship and Table 1 shows that, in spite of a fairly wide scatter, the observed temperature coefficients were of the expected order. Insufficient material was available for detailed checking of these figures and it is not possible to say whether the apparently high value for the 8·2% moisture sample is significant. It should be pointed out, however, that storage at constant moisture content would give a somewhat higher temperature coefficient than storage at constant relative humidity (as in the casein experiments), since the relative humidity for a given water content increases with temperature, although the effect is small at very low water contents (Lea & Hannan, 1949).

Influence of water on the reaction. Table 2 presents data for a wide range of moisture contents, the first four lines being taken from Fig. 1 and the remainder of the table from a subsidiary experiment carried out at constant relative humidities (R.H.'s) of 73, 80 and

Table 1. *Effect of temperature on the rate of loss of amino groups in dried human blood plasma containing added glucose and citrate*

($Q_{10}^{20-30°}$ = rate of reaction at 30°/rate of reaction at 20°.)

Water content (%)	Storage at 20° Days	Storage at 20° Amino-N as % of initial	Days for same change at 37°	$Q_{10}^{20-30°}$
—	0	93·8	—	—
1·9	163	89·4	12	4·8
3·7	163	88·2	9	5·6
8·2	54	82·2	1·5	8·5

85 %. The results are basically similar to those obtained with the casein-glucose system in that the reaction rate is low at low humidities, reaches a maximum at intermediate values and falls off again as the humidity is further increased.

Table 2. *Relation between equilibrium relative humidity, water content and rate of amino-glucose reaction at 37° in dried human blood plasma containing added citrate and glucose*

Relative humidity (%)	Water content (g./100 g. dry non-glucose solids)	mg. Amino-N/g. total N* 4 days	8 days
0	0·0	72·0	71·9
30	5·5	63·6	59·4
42	8·2	53·9	45·2
63	22·6	47·0	40·0
73	37	55·9	47·6
80	52	58·8	52·0
85	69	—	59·1

* Original value, 72·1 mg. amino-N/g. total N.

The maximum rate occurs at about 55 % R.H. which, from Fig. 2, can also be seen to be near the point at which the water relations isotherm begins to

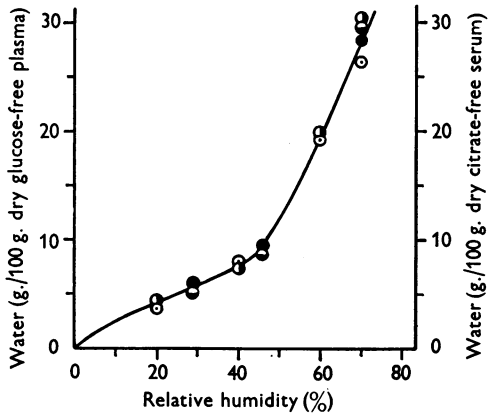

Fig. 2. The water relations at 37° of dried citrated plasma, alone, ●, and with glucose, ⊙; and of dried serum alone, ◒, and with citrate, ◑.

swing rapidly upwards. A similar coincidence was observed with the casein-glucose reaction where, however, it occurred at the higher figure of 65–70 % R.H. (Lea & Hannan, 1949), and it was suggested that water added after this stage is not bound to the protein and slows down the reaction by removing sugar from the surface. A similar mechanism could be pictured for the plasma, but the isotherm differs considerably from those of simple protein systems (see below), and a closer examination of the possible significance is not justifiable at present.

Dried human plasma apparently holds about twice as much water at the higher humidities as do isolated blood proteins or casein (cf. approx. 16 % water at 70 % R.H., quoted by Bull (1944) for various horse-blood albumins and globulins). The reason is obscure: it is not due to the glucose or the citrate added, since approximately the same isotherms were obtained with dried preparations of plasma-citrate-glucose, plasma-citrate, serum-citrate and serum alone (Fig. 2).

Samples containing no added glucose

These samples had initial values of 70·9 mg. amino-N/g. of total N, and 49 mg. glucose/100 ml. plasma corresponding to 0·06 of an equivalent of the amino-N. Fig. 3 shows that the effects observed were much less marked than with the high glucose samples, but the reaction is obviously the same, and the decrease in rate compared with the previous samples was approximately proportional to the decrease in sugar content.

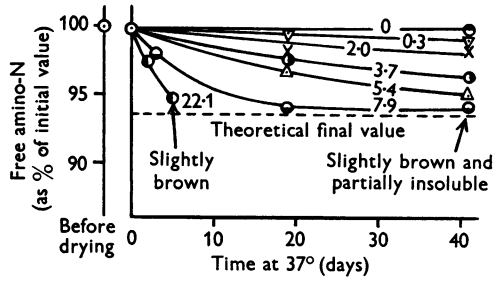

Fig. 3. The loss of free amino-N in dried human blood plasma containing added citrate only on storage at 37° in the presence of the indicated amounts of water (as g./100 g. dry non-glucose solids).

It is interesting to note that, although in these samples dried without extra glucose the reaction during drying was negligible and the total extent of the loss of amino-N even under favourable conditions was small, partial insolubility and brown colour eventually developed after 40 days at 37° and 7·9 % moisture content. The brown colour was relatively less intense than in the corresponding samples containing added glucose, but the insolubility, although small, would be sufficient to render the material unsuitable for transfusion purposes.

DISCUSSION

These experiments exhibit, at all points investigated, a striking similarity to those already recorded in the more extensive experiments with casein-glucose mixtures, and there can be little doubt that basically the same reaction is taking place in both cases. It was shown with the casein that the fall in free amino groups is accompanied by an equimolecular loss of free glucose and an increase in weight of the undialysable protein fraction consistent with a binding by it of all the lost glucose. The stability of this complex was demonstrated by the facts that: (1) pro-

longed dialysis failed to recover free glucose or alter the dry weight of the protein; (2) free amino groups were not regenerated by long exposure either to the acid conditions of the Van Slyke determination or the slightly alkaline conditions of the fluorodinitrobenzene reaction; (3) the lysine residues affected by reaction with glucose became, in large part at least, nutritionally unavailable to young rats. If the plasma proteins behave similarly, some of the samples in the present experiments must have bound up to 5% of their weight of glucose before any visible change developed.

Dried blood plasma as prepared in this country usually has a very low water content and, provided that storage is in completely moisture-proof containers at low temperatures, the extent of the reaction which takes place after drying is unlikely to be large. It would be difficult to reveal small changes by examination of any given sample of stored plasma in the absence of a value for the original free amino-N content of the fresh material. While no survey of stocks of plasma has been attempted, a single bottle which had been stored at laboratory temperature for 5 years was examined. The contents, apparently of normal colour and solubility, showed a water content of 1·9% and a Van Slyke figure of 65·0 mg. amino-N/g. total N (not corrected for urea) whereas three samples of fresh blood gave values of 73·1, 77·7 and 79·1 mg.

The situation is completely different if the container (which is not normally all glass) develops a leak, particularly if the temperature of storage is also high. Even a minute leak, aided by the pumping action of barometric and temperature changes, would be sufficient in time to permit significant amounts of moisture to be absorbed by the hygroscopic contents. That this can happen is shown by the fact that occasional plasma samples have been rejected by users because they were discoloured and failed to reconstitute properly. The present thesis is, however, that long before these obvious signs of deterioration appear, pronounced reaction can have taken place, resulting in the formation of new protein complexes.

The physiological properties of these bodies are as yet unexplored. With nutritive studies, where this reaction has hitherto attracted most attention, the main interest centres round the fact that the linkage resists hydrolysis by the enzymes of the gut (Henry, Kon, Lea & White, 1948), but with dried plasma possible toxic and immunological properties are obviously of prior importance. Harmful effects of this type have not, to our knowledge, been demonstrated, and the present preliminary work is not intended to imply that the existing method of drying plasma is dangerous. It does, however, indicate that the consequences of the protein-sugar reaction in dried plasma should be further investigated and that the practice of adding glucose, which so greatly accelerates deterioration, might well be reconsidered. Further control of deterioration during storage can best be obtained by drying to the lowest practicable moisture content, closing the container so as to avoid all possibility of rehydration, and storing at as low a temperature as possible.

Finally, these experiments also emphasize the care which must be taken in drying biological materials in general when reducing sugars are present. Even in freeze drying, where rapid evaporation keeps the material cold until most of the loosely bound water has been given up, there is a danger that the temperature may rise excessively during that critical period when the water content of the material passes through the region optimal for the protein-sugar reaction.

SUMMARY

1. Dried human plasma as normally prepared contains both citrate and added glucose, and the latter substance may react with the proteins during drying and storage. The amino-N content of the protein falls, and as much as 5% by weight of glucose can be attached to the proteins without any readily apparent change being produced. Insolubility and brown colour eventually develop.

2. The rate and extent of the reaction is determined largely by the water content, and the temperature coefficient is high.

3. Even when the dried plasma contains no added glucose, the small amount normally present can produce the same effects on a greatly reduced scale.

4. The water-holding properties of the dried plasmas deviate considerably from those of simple protein or protein-glucose systems at higher moisture contents.

This work was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research, in collaboration with the Department of Pathology of the University of Cambridge.

REFERENCES

Bull, H. (1944). *J. Amer. chem. Soc.* **66**, 1499.

Greaves, R. I. N. (1946). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 258.

Henry, K. M., Kon, S. K., Lea, C. H. & White, J. C. D. (1948). *J. Dairy Res.* **15**, 292.

Lea, C. H. & Hannan, R. S. (1949). *Biochim. Biophys. Acta*, **3**, 313.

Lea, C. H. & Hannan, R. S. (1950*a*). *Biochim. Biophys. Acta*, **4**, 518.

Lea, C. H. & Hannan, R. S. (1950*b*). *Biochim. Biophys. Acta*, (in the Press).

Lea, C. H. & Hannan, R. S. (1950*c*). *Nature, Lond.*, **165**, 438.

Strumia, M. M. & McGraw, J. J. (1943). *J. Lab. clin. Med.* **28**, 1140.