INFLUENCES OF ERYTHROCYTES AND OF LEUKOCYTES ON STABILITY AND TRANSFER OF ASCORBIC ACID IN HUMAN BLOOD[1]

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Hitherto, in investigations of the stability and distribution of ascorbic acid in blood (1, 2), no attempt has been made to distinguish the relative activities of erythrocytes and leukocytes. These two groups of cells, however, differ with respect to development, structure, function and fate. It therefore seemed desirable to study the influence on and the reaction to the serum ascorbic acid of each type of cells separately. The results of such an investigation are here reported. Some observations on the influence of composition and concentration of serum protein on distribution and stability of serum ascorbic acid are added.

METHODS

Blood from healthy human subjects only was used. For reasons set forth previously (1), no anticoagulant was added, but freshly drawn blood was defibrinated by stirring with a glass rod. In defibrinated blood, leukocytes cannot be separated from erythrocytes as distinctly as in oxalated blood. Therefore, a compromise was worked out on the following basis: Instead of studying red blood corpuscles and leukocytes separately, *i.e.*, each without the presence of the other, erythrocytes with and without leukocytes were observed. Once the effect of the presence of erythrocytes became known, differences in reactions noted with erythrocytes plus leukocytes were ascribed to the presence of the latter.

Defibrinated blood was transferred to ordinary conical 15 cc. centrifuge tubes (Pyrex glass) which were stoppered and kept for 1½ hours at about 25° C. During this time of slow spontaneous sedimentation, leukocytes remain in the upper layer. Thereafter the tubes were centrifuged at moderate speed for 20 minutes. After carefully removing the supernatant serum,[2] a semicapillary pipette was introduced to the bottom of the centrifuge tube and approximately four-fifths of the cells were transferred to a flask by means of suction. This portion of cells was found to contain no leukocytes, or almost none. Serum was added in amounts proportional to the number of erythrocytes desired. The remaining one-fifth of cells was gently shaken with about twice its volume of serum in order to suspend the layer of cells adherent to the wall of the centrifuge tube.

Determination of ascorbic acid

In whole blood, ascorbic acid was determined by the method of Emmerie and van Eekelen (3), in plasma by that of Mindlin and Butler (4).

For the latter procedure, if applied to serum obtained from defibrinated blood, it has been shown previously (1) that a stable color with dichlorindophenol is only developed if the final reaction of the filtrate is so adjusted that 0.215 cc. of 0.1 N NaOH will bring it to the neutral point with phenolphthalein as indicator. From sera with normal protein content filtrates of this normality can be attained when 0.515 N HPO_3 is used for deproteinization. Since one volume of serum is diluted with one volume of distilled water before two volumes of acid are added, the latter becomes diluted once, reducing its normality to $\frac{0.515}{2} = 0.2575$ N. With normal sera, then, $0.2575 - 0.215$ $(= 0.0425)$ constitutes the decrease in normality due to the combination of HPO_3 with protein. If ascorbic acid is to be measured in sera with abnormal protein concentrations, as in some of our experiments, this decrease in normality has to be measured in advance in order to obtain filtrates of 0.215 N. For this purpose, serum is precipitated with 0.515 N HPO_3 and the normality of the filtrate (A) is measured by titration with 0.1 N NaOH. It was found experimentally that the following equation can be applied:

$2(0.2575 - A + 0.215) = 2(0.4725 - A)$ = normality required for precipitation. Transfers of ascorbic acid from serum to cells were estimated as in previously described experiments (2).

Different concentrations of protein in the same serum were obtained by ultracentrifuging for 2 hours at 45,000 r.p.m. (142,000 g.), the tubes being held at an angle of 25° to the vertical.

Attempts to study serum substrates and filtrates by using Lavietes' method of ultrafiltration (5) were unsuccessful because contact with mercury causes significant destruction of ascorbic acid, even under anaerobic conditions and at 7° C.

EXPERIMENTAL RESULTS

Effect of erythrocytes and of leukocytes on stability of serum ascorbic acid. Serum separated

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[2] Serum thus removed must be centrifuged again since it may still contain a few cells.

at once from defibrinated blood was continuously agitated at about 25° C. and at 37° C. in an atmosphere of air. Under such conditions, ascorbic acid was observed to be destroyed in 9 different separated sera at a rate of from 5 to 10 per cent of the original per hour. This is about the same rate reported by Kassan and Roe (6). Figure 1*A* depicts one experiment representative of 8 with essentially identical findings. The presence of erythrocytes invariably protected ascorbic acid in serum, whereas the presence of leukocytes enhanced the rate of destruction.

Transfer of added ascorbic acid from serum to erythrocytes and to leukocytes. Figure 1*B*, representative of 5 experiments, demonstrates that (*1*) no significant decrease in the concentrations of ascorbic acid in true serum was noticeable with erythrocyte counts varying from 3.7 to 4.4 mil-

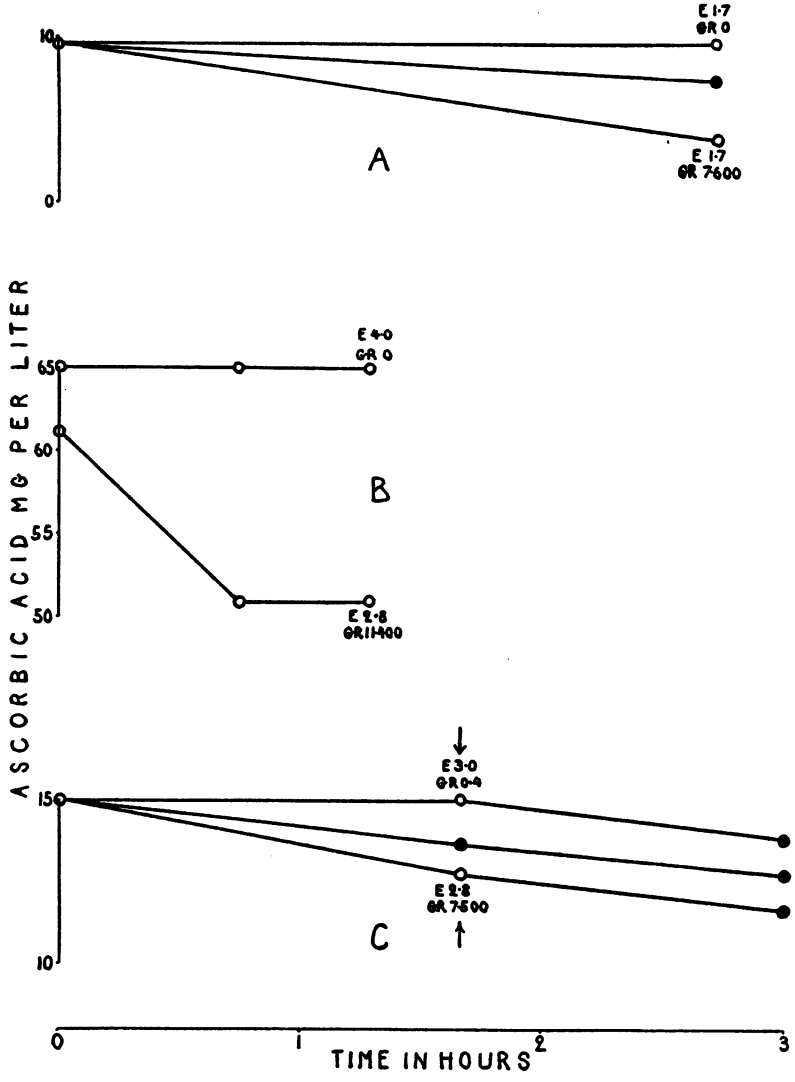

FIG. 1. STABILITY AND TRANSFER OF ASCORBIC ACID IN BLOOD CONTAINING DIFFERENT TYPES OF CELLS

Solid circles indicate sera separated from cells at zero time, open circles indicate sera removed immediately before determinations. All experiments were carried out with continuous agitation; *A* and *C* at room temperature in an atmosphere of air; *B* under nitrogen at 37° C. "E" and "GR" = abbreviations for erythrocytes (in millions) and leukocytes (in thousands).

lions per cubic millimeter and (*2*) when leukocytes were also present, transfers of ascorbic acid regularly occurred and, as previously observed, were self-terminative in about 30 minutes. The amounts transferred were proportional to the number of leukocytes.

Effects of different protein concentrations on distribution and stability of ascorbic acid in serum. Table I presents concentrations of ascorbic acid in different layers obtained from the same serum by ultracentrifuging. It also shows the rate of deterioration of ascorbic acid in each layer when exposed to air for one hour at 28° C.

TABLE I

Distribution and stability of ascorbic acid in serum when protein concentrations were changed by ultracentrifuging

	Upper layer	Middle layer	Lower layer
Total protein, *per cent*	4.46	5.54	7.60
Albumin, *per cent*	3.30	4.26	4.71
Globulin, *per cent*	1.16	1.28	2.89
Ascorbic acid, *mgm. per liter, initial*	13.2	13.4	12.8
After one hour at 28° C. in air	11.8	11.6	11.6

* To 50 cc. of serum from defibrinated blood one cc. 1.5 M phosphate buffer pH 7.3 was added in order to maintain identical pH in all layers.

DISCUSSION

There is unanimity among the students of this subject that ascorbic acid in true serum of whole blood is significantly more stable than in separated serum. The presence of blood cells obviously protects serum ascorbic acid. From our experiments it now appears that the effects of red blood corpuscles and of leukocytes are essentially different. Ascorbic acid is completely protected in true serum containing erythrocytes only.

This stabilizing effect of red cells was also studied with varying numbers of cells. When calculated per one mgm. of ascorbic acid in serum, it appeared quite regularly that approximately 100,000 erythrocytes per cubic millimeter do protect such a concentration, while fewer cells do not.

Under the same conditions, *viz.* in air, leukocytes do not prevent, but rather enhance the deterioration of ascorbic acid in serum. Determinations of ascorbic acid in whole blood carried out simultaneously proved that this decrease of serum ascorbic acid in air in the presence of leukocytes is due to actual loss and not to oxidation of ascorbic acid to its dehydro form. This effect of leukocytes obviously requires the presence of oxygen, since it did not occur under nitrogen. From these observations it would appear that erythrocytes and leukocytes affect oxidative and/or reducing processes in serum in essentially different ways. Neither of these influences seems to be due to changes in serum induced by cellular leakage; they seem rather to be dependent upon the presence of intact cells. In Figure 1*C* erythrocytes again had protected serum ascorbic acid, while erythrocytes plus leukocytes had promoted its deterioration up to the time indicated by arrows when both these sera were removed from their respective cells and further observed as separated sera. It then became evident that their rate of deterioration followed rather closely that of serum separated at the very beginning of the experiment.

Under these conditions of our experiments, ascorbic acid was transferred from serum to leukocytes but not to erythrocytes. This observation does not imply that erythrocytes do not contain ascorbic acid *in vivo*. It is quite possible that observations extended over longer periods of time would establish transfer of ascorbic acid from serum to erythrocytes, too. Such an extension of the duration of observation, however, involves the danger of cell volume changes or hemolysis which are prohibitive in studies of distribution. But even if transfer from serum to erythrocytes could be demonstrated *in vitro,* the amounts transferred could only be a fraction of those transferred to leukocytes.

A similarly significant difference between the ascorbic acid content of leukocytes and erythrocytes was described by Butler and Cushman (*7*) who noted a concentration of ascorbic acid in the former of about 40 times that in the latter. Crandon, Lund and Dill (*8*) confirmed this observation and further reported that leukocytes retain their ascorbic acid much longer than erythrocytes in the course of vitamin C deprivation. Our findings that *in vitro* transfer of ascorbic acid occurs chiefly from serum to leukocytes is in complete agreement with *in vivo* observations by Ralli

and Sherry (9). These authors noted decreased plasma concentrations of ascorbic acid following the administration of insulin. Upon further investigation, this decrease appeared to be due to a redistribution of serum ascorbic acid rather than to true loss. The decrease in plasma ascorbic acid, then, was found to be associated with a rise in the ascorbic acid content of leukocytes (and platelets), whereas no such increase could be demonstrated for red cells.

As in previously reported experiments (1, 2), transfers were studied at 37° C., with continuous agitation, in an atmosphere of nitrogen, the latter being essential for the prevention of oxidative destruction. The protective action of nitrogen was reestablished not only for separated sera but also for true serum separated after 45 and 90 minutes from blood containing only 1.4 million erythrocytes, but 6,800 leukocytes per cubic millimeter, respectively. As stated above, the latter type of cells caused increased destruction of serum ascorbic acid in air; since erythrocytes protected serum ascorbic acid even in air, transfer of ascorbic acid added to serum could also be studied in such an atmosphere with blood containing only erythrocytes. No transfer of ascorbic acid from serum to erythrocytes up to 5.0 million per cubic millimeter was detected in blood exposed to air.

This striking difference between erythrocytes and leukocytes in their reaction to large concentrations of serum ascorbic acid further supports earlier conclusions that the distribution of ascorbic acid in blood does not follow the laws of simple diffusion. It was demonstrated previously (1, 2) that the exchange of ascorbic acid between serum and cells (*a*) depends on the maintenance of physiological temperature, (*b*) is unidirectional, *viz.*, from serum to cells only and not vice versa; from the presently reported experiments it can be added that this exchange (*c*) involves only leukocytes. In other tissues, too, preferences similar to these observed among blood cells seem to prevail; the endocrine glands, *e.g.*, the adrenals, are recognized as containing significantly higher ascorbic acid concentrations than liver, kidney or muscle tissues.

Our observations that changes in protein content, subsequent to ultracentrifuging, did not affect the distribution of ascorbic acid in serum confirm findings by Coolidge (10) who mentions equal concentrations of ascorbic acid in substrate and ultrafiltrate of human serum obtained by his newly devised method.

The previously established irreversibility of the transfer of ascorbic acid from serum to cells (2) had been attributed to a combination of ascorbic acid with intracellular substances. In that connection, proteins had been mentioned because several communications referred to ascorbic acid as bound to this group. From our present studies, however, it is obvious that ascorbic acid is not bound to serum proteins. Nor did different concentrations or differences in composition of serum protein influence the stability of ascorbic acid when studied in an atmosphere of air.

SUMMARY

In an atmosphere of air, the deterioration of serum ascorbic acid is prevented by the presence of intact erythrocytes, while it is enhanced by that of leukocytes.

Under nitrogen, ascorbic acid added to serum is transferred to leukocytes but not to erythrocytes. Neither do the latter take up the vitamin in an atmosphere of air.

Ascorbic acid is not bound to serum proteins nor do different protein concentrations induce different rates of deterioration of serum ascorbic acid.

The influence of leukocytes was not studied separately but was estimated from studies of erythrocytes with and without leukocytes, the differences between the two being ascribed to leukocytes.

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