The Effects of Various EDTA Complexes on Coagulation

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DISODIUM ETHYLENEDIAMINE TETRAACETATE (EDTA) has been known for some time to prolong coagulation in vitro.[1-3] During the course of treatment of seven patients for lead intoxication with disodium calcium EDTA, we noted no hemorrhagic manifestations.[4, 5] However, Wishinsky[6] described hypoprothrombinemia appearing in a patient treated with disodium calcium EDTA for hemochromatosis. Recently Dudley et al.[7] reported renal hemorrhage at autopsy of two patients with hypercalcemia treated with disodium EDTA. One of these patients had a prothrombin level of 50 per cent during the course of her therapy. Chemical studies of the heart blood of one case demonstrated a high fibrinogen level and inability of a clot to form despite an excess of calcium chloride. A stable clot formed, however, when prothrombin was added and when normal plasma with 100 per cent prothrombin activity was added to the patient's plasma, resulting in a prothrombin activity of the mixture of 50 per cent of normal. Neither fibrinolysin nor heparin activity could be demonstrated in this blood.

The anticoagulant properties of EDTA have been studied by several authors. Dyckerhoff et al.[8] ascribed them to antithrombic and, to a lesser degree to antiprothrombic effects. Zucker[9] demonstrated that the one-stage prothrombin time was prolonged, but that the two-stage method gave normal values after the addition of beef serum, indicating that prolongation in the one-stage method was probably due to destruction of Factor V. These findings were corroborated by Triantaphyllopoulous, Quick and Greenwalt.[10] Zucker suggested that a partial explanation of these observed effects might be correlated with the removal by EDTA from plasma of ions other than calcium and she suggested that magnesium might be implicated. This report indicates that a metal is probably removed by EDTA, but that magnesium is not involved.

Material and Methods

Oxalated plasma was prepared by mixing one volume of 0.1 M sodium oxalate with nine parts of whole blood and centrifuging this mixture at 1,700 revolutions per minute for 10 minutes. Prothrombin determinations were performed in duplicate by the one-stage method of Quick.[11] Only those values which agreed within 10 per cent were used. The source of thromboplastin was commercial (Difco) rabbit brain thromboplastin, and .02 M calcium chloride was used for recalcification. Prothrombin-free plasma was made by incubating 100 mg. of barium sulfate (Baker) with 1 ml. of oxalated plasma for 10 minutes at 37 C. A prothrombin activity curve was made by pooling the plasma of five normal adult donors and performing one-stage prothrombin times on increasing percentages of this plasma diluted with prothrombin-free plasma. This was done in duplicate on two occasions and the average

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of these two determinations used for a final value. The curve produced was similar to that of Alexander et al.[12] for prothrombin activity. EDTA complexes of various metals were made by reacting equimolar amounts of the metal carbonates or oxides with EDTA and either bringing the solutions to volume or evaporating them to dryness and using the dry powder to make up a standard solution containing the equivalent of 100 mg. Na_2 EDTA per ml. To prevent dilution of the plasma these substances were added in the following manner: 0.02 ml. of the solution was dried at 100 C in the bottom of a test tube. To perform the test 2 ml. of oxalated plasma were added to the tube and the dried material was dissolved in the plasma. The amounts of EDTA complexes which were added were sufficiently small so that no difficulty was encountered in effecting solution in the plasma.

COMPARISON OF DISODIUM EDTA AND DISODIUM CALCIUM EDTA

If EDTA prolongs the prothrombin time by removing a metal, then EDTA saturated with the metal removed should have no effect on the prothrombin time. The first metal to be so tested was calcium.

Na_2 EDTA and $CaNa_2$ EDTA were incubated with oxalated plasma at 37 C. at a level of 1.0 mg. Na_2 EDTA per ml. The prothrombin times determined at 3 hours and at 7.5 hours are recorded in table 1.

This was repeated at 5 C. because there appeared to be a difference between the disodium salt and the calcium complex which was removed on prolonged incubation. These results are shown in table 2.

The difference in the effect of disodium calcium EDTA and disodium EDTA on the prothrombin time of oxalated plasma at three hours, at 37 C. is magnified by incubation at 5 C.

Although the incubation at a lower temperature partially prevented the loss

TABLE 1.—*Incubation of Oxalated Plasma at 37 Ca. with Disodium Calcium and Disodium EDTA for 3 and 7.5 Hours*

Untreated	Disodium calcium EDTA	Disodium EDTA
Incubation for 3 hours		
Undiluted 15.2	17.7	28.0
Incubation for 7.5 hours		
25.8	33.3	33.0

TABLE 2.—*Incubation of Oxalated Plasma with Disodium Calcium EDTA and Disodium EDTA at 5 C.*

Untreated	Disodium calcium EDTA	Disodium EDTA
Incubation for 3 hours		
14.0	13.9	21.2
Incubation for 8 hours		
15.4	17.3	25.8

of prothrombin activity, there was still a definite effect of the calcium complex. This indicated that the metal removed was not calcium. The lower temperature probably slowed the equilibration of CaNa EDTA with labile factor. Since the saturation of the EDTA with Calcium did not remove its effect, the possibility that another metal was involved in the prothrombin transformation was considered. Rough calculation of the temperature coefficient of this effect of EDTA suggests a physical rather than an enzymatic reaction. Studies are in progress to clarify this point.

The effect of other polyvalent metals was studied using EDTA complexes at a concentration equivalent to 1 mg./ml. of Na_2 EDTA.

The results are shown in table 3a and table 3b.

It will be seen from the tables that a marked prolongation of the prothrombin time of oxalated plasma occurred on incubation with disodium, copper, ferrous, stannous, cobalt and magnesium EDTA. Further, the addition of prothrombin-free plasma partially or completely corrected the prothrombin time. From the values in parenthesis which represent residual "prothrombin activity," it can be seen that there was a significant difference between the effect of these complexes and the lack of effect of the complexes of nickel, manganese and zinc. The difference between these two groups of complexes was consistent over repeated determinations with many different specimens of plasma.

These observations show that some metal EDTA complexes, but not all, produce a prolongation of the prothrombin time due to a destruction of inhibition of Ac-Globulin (Factor V, labile factor) as suggested by Zucker[9] and by Triantaphyllopoulous et al.[10] It would seem that this destruction of inhibition may be due to the chelation of some ion or ions which may be concerned with the maintenance of Ac-Globulin or its conversion into an active form.[13] Zucker[9] suggested that it might be magnesium; however, oxalated plasma incubated with magnesium EDTA underwent a prolongation of the prothrombin time. This shows that magnesium is not the metal removed when EDTA prolongs the pro-

TABLE 3a.—*One-Stage Prothrombin Determinations on Oxalated Plasmas Incubated for 7 Hours* ($MeNa_2EDTA$)

Complex	Undiluted	1/4 dilution with prothrombin-free plasma
Control	21.1 sec. (20%)	19.9 sec.
Na_2 EDTA	99.3 (5%)	23.4
MgK_2 EDTA	38.3 (7%)	23.8
$ZnNa_2$ EDTA	24.9 (15%)	20.8
$CuNa_2$ EDTA	55.5 (5%)	22.0
$FeNa_2$ EDTA	153.0 (<5%)	20.7
$SnNa_2$ EDTA	230.5 (<5%)	21.5
$MnNa_2$ EDTA	27.0 (13%)	21.4

TABLE 3b

Control	16.9 (35%)	17.3
Ni EDTA	16.7 (35%)	18.4
Co EDTA	26.7 (13%)	19.9

thrombin time (see table 3). Since several metal EDTA complexes produced only a negligible effect on the prothrombin time, one of two possibilities is suggested. If the EDTA metal complex is so tightly bound that no free EDTA is available for removing the crucial metal from the Ac-Globulin complex, then the metal EDTA attached to the EDTA is the metal which is normally involved with Ac-Globulin, then this metal EDTA complex will also have no effect on the prothrombin time.

The effective complexes which potentiate the disappearance of Ac-Globulin can be explained on the basis of the relative affinity of the chelating metal to the EDTA compared with the affinity of the metal of the Ac-Globulin complex to the EDTA. This is expressed in the following equilibrium:

(1) Me_1 EDTA + Me_2 Ac-Globulin (active) $\rightleftarrows$ Me_2 EDTA + Me_1 Ac-Globulin (inactive).

The position of this equilibrium would thus be determined by the affinity constants of the various complexes. A further question is posed by this equilibrium; namely, is the metal Ac-Globulin complex stable or does the new metal hasten the irreversible denaturation of the protein? A second possibility is that the added metal in the EDTA complex is inert, and that Ac-Globulin is only active in the presence of a particular essential metal, which is removed by the traces of uncomplexed EDTA, arising from the dissociation of the added complex. This can be expressed by the following equilibrium:

(2) Me_1 Ac-Globulin (active) + Me_2 EDTA $\rightleftarrows$ Ac-Globulin (inactive) + Me_1 EDTA + Me_2.

To explore these possibilities one-stage prothrombin times were determined on oxalated plasma incubated with metal salts at a concentration of .001 M at 37 C. These are shown in tables 4 and 5.

The incubation of most metals tested produced a prolongation of the pro-

TABLE 4.—*Incubation of Various Metals with Oxalated Plasma for 6.5 and 21 Hours*

	Untreated	$CoAc_2$	$MnCl_2$	$CuSO_4$
Incubated for 65 hours				
Undiluted	20.7	180	21.0	20.5
1/4 dilution with PFP	17.0	39.0	19.5	19.1
Incubated for 21 hours				
Undiluted	33.8	180	34.7	99.3
1/4 dilution with PFP	23.5	86	25.1	27.6

TABLE 5.—*Incubation of Various Metals for 6 Hours at 37.5 C.*

	Untreated	$BaCl_2$	$NiSO_4$	$FeSO_4$	$FeCl_3$
Undiluted	19.5	19.3	15.8	33.3	30.2
1/4 dilution with PFP	17.2	16.9	16.0	18.7	18.6

TABLE 6

	Untreated	0.1 M	0.01 M	0.005 M	.001 M	.0001 M	.00001 M
Undiluted	11.9	Incoag.	23.7	16.1	11.8	12.1	12.3
1/4 dilution with PFP	17.7	Incoag.	25.2	18.2	16.8	17.2	18.0
Incubation for 6 hours							
Undiluted	19.0	Incoag.	42.1	22.0	14.6	18.4	17.8
1/4 dilution with PFP	18.4	Incoag.	28.7	19.8	18.0	18.3	16.4
Incubation for 24 hours							
Undiluted	26.9	Incoag.	Incoag.	4 mins.	55.7	85.1	—
1/4 dilution with PFP	18.5	Incoag.	Incoag.	2 mins., 40 secs.	22.6	20.2	—

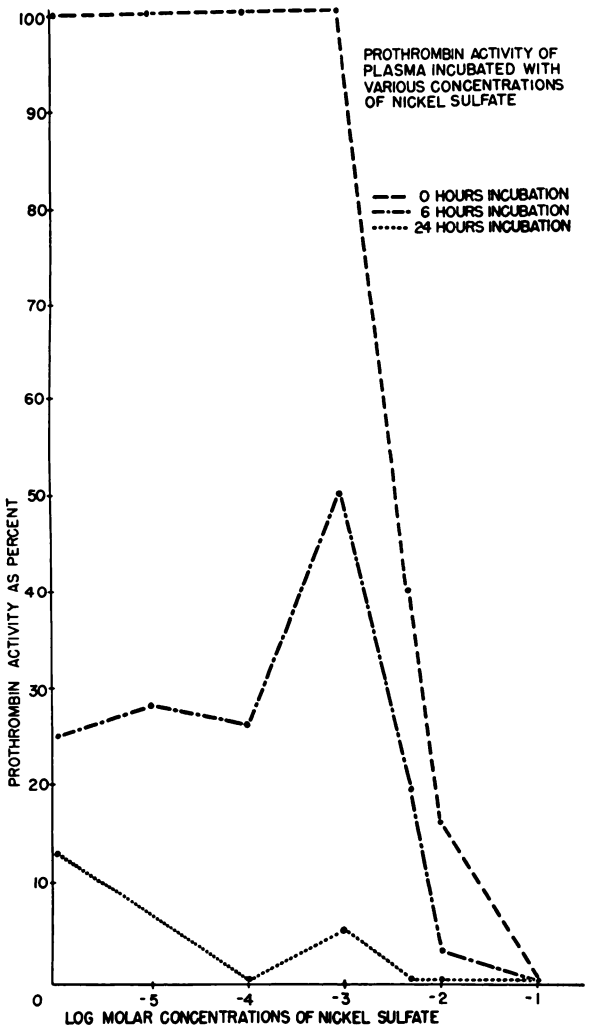

FIGURE 1.

thrombin time by the one-stage method. In the case of cobalt acetate, this effect does not appear to be on Ac-Globulin as the addition of prothrombin-free plasma did not bring the prothrombin time to the untreated level. Further studies concerning this metal are now in progress. Manganese, zinc, nickel, and barium did not affect the prothrombin time under test conditions. Nickel seemed to preserve the Ac-Globulin activity on repeated tests. To determine the maximal effective concentration of nickel, various dilutions were made of nickel sulfate and oxalated plasma, and these were incubated for 6 and 24 hours. The results are presented in table 6 and figure 1.

This shows that .001 M nickel sulfate was the maximal effective concentration for shortening the prothrombin time, whereas at a higher concentration, nickel prolonged the prothrombin time. This prolongation was not affected by the addition of prothrombin-free plasma which would suggest that at these higher concentrations prothrombin was affected, rather than Ac-Globulin.

Discussion

Incubation of Na_2 EDTA or certain of its metal chelates with plasma produced a prolongation of the prothrombin time as determined by the one-stage method. This prolongation was partially or completely corrected by the addition of prothrombin free human plasma. This suggests that the chelation of certain metals in the plasma causes a destruction or inhibition of action of Ac-Globulin, its precursor substances, or activators.

The incubation of plasma with certain metal salts at .001 M had diverse effects. In the case of manganese and barium there was no difference between the untreated oxalated specimen and that incubated with these metals. Ferric, ferrous and cobaltous ions produced a prolongation of the prothrombin time. In the case of cobalt, this did not appear to be an effect concerned particularly with Ac-Globulin because the control prothrombin activity could not be restored by addition of prothrombin free plasma. These various metal ions were tested at .001 M and the EDTA complexes at .003 M. These concentrations were considerably below the range of nonspecific salt effects on the prothrombin time reported by Loomis'[14] and Phillips'[15] groups.

From the data reported, together with the reports of Zucker[9] and Triantaphyllopoulous et al.,[10] it appears that a metal is involved in the preservation of, or activation of, Ac-Globulin. We have further been able to show that this metal is not calcium, cobalt, magnesium, iron or copper. Zinc, manganese, barium and nickel, since they do not prolong the prothrombin time either in their free or chelated states, belong in a special class and cannot be related to the Ac-Globulin metal in a positive or negative manner with the data now at hand. Any one of these metals could be the essential one, with the reservation that nickel at .001 M concentration seems to protect the Ac-Globulin, whereas the others have no special effect. A definite statement regarding the role of nickel must await the results of further investigations.

A word should be said about the relative affinities of the various metals for EDTA. Table 7 gives a list of stability constants of the complexes of the metals reported in this paper.[16]

The metals with the high stability constants replace those with lower constants in complexes with EDTA. It is interesting to note that if nickel were

TABLE 7.—*The Stability Constants*

Ni++	18.4	Fe_2++	13.4
Cu++	18.3	Ca++	10.6
Co++	16.1	Mg++	8.7
Zn+	16.1	Ba++	2.07
Mn++	13.4	Na+	1.7

$$K = \frac{(Me\ EDTA)}{(Me)\ (EDTA)}.$$

associated with Ac-Globulin it would be removed from the Ac-Globulin by the EDTA complex of any metal with a lower stability constant. These affinity constants were determined on long equilibration times and in pure solutions. Proteins and extraneous ions could alter the magnitude of the affinity constants, and the duration of time required for the attainment of equilibrium. This table can, therefore, serve only as a rough guide. The results with the free metal ions are of greater final significance in the elucidation of the role of metals in the prothrombin mechanism.

SUMMARY AND CONCLUSIONS

The one-stage prothrombin time of oxalated plasma was prolonged on incubation with disodium EDTA. This was due to the destruction of Ac-Globulin or the inhibition of its production from a precursor stage. There was a similar change with disodium calcium EDTA, indicating some metal other than calcium was probably being chelated.

A marked prolongation of the prothrombin time of oxalated plasma occurred on incubation with cupric, ferrous, stannous, cobaltous, and magnesium EDTA, but manganous, zinc and nickel EDTA produced no prolongation of the prothrombin time. This would support the concept that a metal ion may be concerned with the maintenance of Ac-Globulin or its conversion into an active form.

The cobaltous, cupric, ferrous and ferric ions produced a prolongation of the prothrombin time. This effect was corrected by the use of prothrombin free plasma except in the case of cobalt. The manganous, barium and nickel ions did not prolong the prothrombin time and nickel was effective in maintaining the prothrombin time at its original level. Its most effective concentration was .001 M. At higher concentrations it produced a prolongation which was not corrected by the addition of prothrombin-free plasma.

SUMMARIO IN INTERLINGUA

Le uniphasic tempore prothrombinic de plasma oxalate esseva prolongate per incubation con binatrium-EDTA. Isto esseva debite al destruction de globulina Ac o le inhibition de su production ex un stadio precursori. Esseva constatate un simile alteration con binatrium-calcium-EDTA, lo que indicava que un metallo altere que calcium esseva implicate in le processo de chelation.

Un marcate prolongation del tempore prothrombinic de plasma oxalate occurreva post incubation con EDTA cupric, ferrose, stannose, cobaltose, e a magnesium, sed EDTA manganose, a zinc, e nickelose resultava in nulle pro-

longation del tempore prothrombinic. Isto pare supportar le conception que un ion metallic es possibilemente concernite con le mantenentia de globulina Ac o su conversion in un forma active.

Le iones cobaltose, cupric, ferrose, e ferric produceva un prolongation del tempore prothrombinic. Iste effecto esseva corrigite per le uso de plasma libere de prothrombina, excepte in le caso de cobalt. Le iones manganose, a barium, e nickelose non prolongava le tempore prothrombinic, e nickel effectuava le mantenentia del tempore prothrombinic a su nivello original. Su plus efficace nivello esseva 0,001 M. A plus alte concentrationes illo produceva un prolongation que non esseva corrigite per le addition de plasma libere de prothrombina.

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