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THE CHEMICAL STATE OF THE CALCIUM REACTING IN THE COAGULATION OF BLOOD*

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For nearly a half century almost every student of coagulation has accepted the hypothesis that ionized calcium is essential for coagulation. Many investigators, moreover, hold that ionized calcium acts as a catalyst. The origin of these concepts can be traced first to Arthus and Pagès (1) who in 1890 showed that blood was made incoagulable by sodium oxalate and that readdition of calcium restored normal coagulation. They justifiably concluded that calcium was necessary for the coagulation reaction. Two years later Pekelharing (11) found that sodium citrate was equally effective as an anticoagulant, and, being no doubt influenced by the work of Arthus and Pagès, he concluded that the action of sodium citrate was on the calcium. Interestingly, Alexander Schmidt (18) refused to accept the idea that calcium had a specific rôle in coagulation. He attributed the action of both sodium oxalate and sodium citrate to a non-specific salt effect.

Considerable uncertainty remained until Sabbatani (17) explained the peculiar behavior of sodium citrate by stating that calcium forms a complex with this compound whereby its ionization is depressed, and he postulated that ionized calcium is essential for coagulation.

Since Hammarsten (4) had previously found that calcium was necessary for the conversion of prothrombin to thrombin, it became generally accepted that ionized calcium was required for the activation of prothrombin. Morawitz (8) and Fuld and Spiro (3) incorporated the concept that ionized calcium acted catalytically in their "classical theory" of coagulation. Thereafter few investigators had the temerity or sufficiently unconventional minds to question the indispensability of ionized calcium.

It is well established that part of the plasma calcium is free or ionized and part is bound and non-diffusible. It has been estimated by various methods that normally about one-half of the total calcium is bound or unionized. When Vines (24) found that only one-seventeenth of the total calcium present in serum was required to bring about a normal coagulation time of decalcified

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blood, he concluded that only bound calcium participated in coagulation. Scott and Chamberlain (19) reported similar findings. More recently Nordbö (9) found that 0.15 to 0.18 mM of calcium per liter of plasma was necessary to cause coagulation. This amount of calcium is only one-fifteenth of the total found in plasma; nevertheless, he assumes that this fraction is ionized, because it was ionized calcium which he added to decalcified plasma. Ransmeier and McLean (16) determined that the minimal Ca^{++} concentration required for the coagulation of citrated plasma was 0.28 mM per liter of human plasma and 0.18 mM per liter of dog plasma. These values are similar to those of Nordbö who worked with beef and horse plasma. In both studies it was assumed that it was the minimum amount of calcium required to initiate coagulation that was significant.

The introduction by Steinberg (22) of a resin, amberlite, which quantitatively removes calcium from the blood, has made possible a new approach to the problem of calcium in coagulation. By employing this new reagent, Quick (14) found that the minimum amount of calcium required to produce a normal prothrombin time, which is the real criterion of maximum and optimal coagulation, is 0.0012 M for human and 0.0004 M for dog plasma. It was found (15) further that if the amount of calcium added is below these levels, the amount of prothrombin consumed or converted is quantitatively reduced even though thromboplastin is in excess. Neither of these findings are in accord with the theory that calcium acts ionically and plays the rôle of a catalyst.

From this short historical review it should be obvious that the concept that it is ionized calcium in the rôle of a catalyst which is responsible for the activation of prothrombin, is still a theory and not an established fact. Its main support is the assumption that sodium citrate acts as an anticoagulant by depressing the ionization of calcium. It is the purpose of this paper to reexamine critically the rôle of calcium in the coagulation mechanism by three different approaches: (1) the decalcifying action of sodium oxalate; (2) the anticoagulant action of sodium citrate; and (3) the influence of calcium on the stability of the labile factor of prothrombin.

EXPERIMENTAL

Determination of Prothrombin Time

The method of Quick was used. The thromboplastin prepared by dehydration of rabbit brain with acetone consistently yielded a prothrombin time of 11½ to 12 seconds for human blood.

Preparation of Stable Native Plasma

Blood was collected by venipuncture using a needle and syringe coated with silicone (General Electric dri film 9987). The syringe was kept in ice prior to use, and the blood was immediately transferred to silicone-coated tubes immersed in an ice bath,

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and covered with mineral oil. The chilled blood was centrifuged for 15 minutes at 4,000 R.P.M. in an angle centrifuge at refrigerator temperature. The plasma was removed with a silicone-coated pipette and transferred to a silicone-coated test tube.

Preparation of Tricalcium Phosphate

To a solution containing 158 gm. of trisodium phosphate in 1 liter of distilled water, an equal volume of calcium chloride solution containing 66.6 gm. of the anhydrous salt was added slowly with vigorous stirring. The pH was adjusted to 7. The precipitate, $Ca_3(PO_4)_2$, was washed by decantation until the sodium chloride was removed. The suspension was made up to 1 liter and it therefore had a concentration of 0.2 M. From this stock suspension, a 0.008 M preparation was made by diluting 4 cc. with 96 cc. of distilled water. One cc. of the dilute suspension was transferred to a small test tube which was centrifuged to pack the tricalcium phosphate. The water was poured off, the tube drained, and 1 cc. of plasma added. The tricalcium phosphate and plasma were mixed and allowed to stand at room temperature for 10 minutes. The adsorbent was removed by centrifugation.

The Removal of Calcium with Amberlite

The method as outlined by Quick (14) and Stefanini (21) was used.

Quantitative Measurement of the Action of Sodium Oxalate on Prothrombin Activity

Stewart and Percival (23) and later Nordbö obtained evidence that the sodium oxalate did not inhibit coagulation instantaneously, but required considerable time. In 1940 Quick (12) studied this action quantitatively by measuring the increase of the prothrombin time of plasma after the addition of an accurately measured amount of sodium oxalate. He found that the speed of inhibition increased in proportion to the amount of oxalate added. Because of the importance of these results, the experiment was repeated with greater precision and on a larger number of plasmas. The data are presented in Table I.

It will be observed that the addition of 0.02 cc. of 0.1 M sodium oxalate to 1 cc. of human plasma caused only a slow increase in the prothrombin time. Yet, this amount of sodium oxalate brings the concentration to 0.002 M in the plasma. Since the calcium level averages 10 mg. per 100 cc. which is 0.0025 M, the amount of sodium oxalate added is sufficient to precipitate approximately 80 per cent of the total calcium. Since only half of the calcium is ionized and since this fraction is rapidly removed as insoluble calcium oxalate, it appears unlikely that the slow increase in prothrombin time is due to the removal of ionized calcium. Even when the sodium oxalate is increased to 0.004 M (by adding 0.04 cc. to 1 cc. of plasma) which is 60 per cent more than is required to precipitate the total calcium, the diminution of prothrombin activity is slow and one-half hour after the oxalate is added the plasma still clots in 330

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seconds. Under these conditions, the ionized calcium can come only from dissolved calcium oxalate. Since the solubility is less than 0.0006 gm. per 100 cc., the concentration of ionized calcium is below 0.000004 M, which is far less than the values calculated as minimal by Nordbö and by Ransmeier and McLean.

The most logical explanation for the action of sodium oxalate is that instead of only precipitating ionized calcium, it also removes calcium from a compound which is essential for coagulation. Apparently this compound has a great avidity for calcium and therefore a relatively large excess of sodium oxalate is required to decalcify it completely. An examination of the data of Table I

TABLE I

The Speed of Inhibition of the Prothrombin Activity by Sodium Oxalate and Sodium Citrate

	Prothrombin time					
Time after mixing	0	10 sec.	1 min.	5 min.	10 min.	30 min.
	sec.	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
Amount of reagent added to 1 cc. of plasma						
Sodium oxalate 0.1 M						
0.02 cc.	12	12	12½	14	18	*
0.04 cc.	12	12	18	52	165	330
0.06 cc.	12	17	75	∞		
Sodium citrate 0.1 M						
0.02 cc.	12	12½	12½	*		
0.04 cc.	12	15	15	14	13	*
0.06 cc.	12	28	28	27½	25	*
0.08 cc.	12	135	150	135	135	125

* Spontaneous coagulation.

clearly indicates that the reaction follows the law of mass action. Since this compound has a great affinity for calcium, it is logical to assume that when ionized calcium is added to oxalated or otherwise decalcified plasma, a prompt resynthesis of this calcium compound occurs with an immediate restoration of the coagulability of the plasma especially if an excess of thromboplastin is present. This temporary coagulability in the presence of sodium oxalate has been interpreted by both Nordbö (9) and Owren (10) as due to the added ionized calcium remaining as such for a short period. Quick (14), however, showed that this activity remains considerably longer than the time required for the complete precipitation of ionized calcium as the oxalate salt.

It is difficult to find a simpler and more obvious explanation for the slow inhibitory action of sodium oxalate than the one offered; namely, that it re-

moves calcium from a compound which is essential in the conversion of prothrombin to thrombin, but which is inactive in its decalcified state. Sodium citrate appears to act in an entirely different manner. It exerts its maximum depressing action quickly and it only gradually becomes more inhibitory as its concentration is increased. The nature of the action of sodium citrate is shown in the following experiments.

The Action of Sodium Citrate on Prothrombin

Bordet and Delange (2) discovered that tricalcium phosphate removes by adsorption a plasma constituent essential for coagulation. They concluded that it was serozyme (prothrombin). Subsequently other agents such as barium sulfate, magnesium hydroxide, and aluminum hydroxide were found which likewise remove prothrombin by adsorption. It was generally accepted

TABLE II

The Blocking Action of Sodium Citrate on the Absorption of Prothrombin by Tricalcium Phosphate

Molarity of sodium citrate of which 1 volume was added to 9 volumes of plasma....	Prothrombin time after absorption with $Ca_3(PO_4)_2$										
	Control*	0.25	0.20	0.15	0.12	0.10	0.08‡	0.06‡	0.04‡	0.02‡	0
	sec.	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
Rabbit.....	7	7	7½	10	11½	13½	17	18	21	25	∞
Dog.....	5½	5½	5½	5½	5½	6	8	10	18	48	∞
Man.....	12	12	12	12	12	13½	15½	19	33	135	∞

* Control. Prothrombin time of oxalated plasma not treated with $Ca_3(PO_4)_2$.

‡ For low concentrations of sodium citrate, oxalated plasma in place of native plasma was used to prevent spontaneous coagulation.

that the prothrombin thus removed was a unitary substance which required only thromboplasin and calcium for its activation. This concept lost its validity when in 1943 Quick (13) found that prothrombin, or perhaps more accurately, the chemical aggregate on which prothrombin activity depends, consists of a complex, only part of which is adsorbable by aluminum hydroxide.

To avoid confusion, however, the term prothrombin, will be reserved in this paper for the adsorbable fraction while the designation of the labile factor will be applied to the non-adsorbable part of the complex which diminishes when plasma is stored.

The adsorption of prothrombin by tricalcium phosphate which occurs readily from oxalated plasma fails to take place when sodium citrate is used. There is moreover a definite quantitative relationship between the concentration of sodium citrate and the non-adsorbability of prothrombin. This relationship was studied and the results obtained are recorded in Table II. It can be seen that enough sodium citrate must be added to human blood to produce a final

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concentration of a little more than 0.01 M before all the prothrombin remains non-adsorbable. Below that concentration some of the prothrombin is removed by tricalcium phosphate and therefore the prothrombin time becomes prolonged. Rabbit blood requires a somewhat higher concentration of sodium citrate, approximately 0.02 M to prevent completely adsorption of prothrombin, whereas dog blood is similar to human blood in its response to sodium citrate.

This finding clearly suggests that sodium citrate combines with prothrombin and thereby blocks its adsorption with agents such as tricalcium phosphate. Such a union obviously does not occur between prothrombin and the oxalate radical. To rule out the possibility that prothrombin itself is not adsorbable but may become so after decalcification by sodium oxalate, several studies (recorded in Table III) were made. Thus, native hemophilic plasma when treated with tricalcium phosphate loses all prothrombin activity, but if mixed

TABLE III

Prothrombin Time of Plasma (in Seconds)

	Native	Amberlite-treated	Oxalated
Control*	12	12	12
After treatment with $Ca_3(PO_4)_2$	∞	∞	∞
After addition of sodium citrate and treatment with $Ca_3(PO_4)_2$‡	12	12	12

* Control. 0.1 cc. of plasma, 0.1 cc. of thromboplastin, and 0.1 cc. of 0.02 M $CaCl_2$.

‡ To 0.9 cc. of plasma, 0.1 cc. of 0.2 M sodium citrate was added.

with sodium citrate before the addition of the adsorbing agent, it retains its prothrombin completely. Blood treated with amberlite, the agent that removes calcium and so renders it incoagulable, will when treated with tricalcium phosphate lose its prothrombin activity. If however, sodium citrate is added, the adsorbent no longer is able to take out the prothrombin. In fact the adsorption is blocked even in oxalated plasma if sodium citrate in sufficient amounts is added (Tables II and III). The removal of prothrombin by tricalcium phosphate is therefore independent of calcium.

The conclusion that prothrombin forms a union with sodium citrate and thereby loses its adsorbability can be accepted without hesitation, but the moot question remaining is: does this combination with citrate cause the prothrombin to become inactive? In other words, is sodium citrate an antiprothrombin? The difficulty in arriving at a solution of this problem is that calcium also forms a complex with the citrate radical, as a result of which calcium ionization is depressed. If one considers the low Ca^{++} in the oxalated plasma of Table I which still permits coagulation, it seems unlikely that the concentration of

citrate required to inhibit coagulation is sufficient to depress Ca^{++} below this effective level since the primary dissociation constant of calcium citrate is fairly high. According to Hastings, McLean, and their associates (5) the $pK_{Ca\ citrate}$ is 3.22. Further work will, however, be required before a final answer can be given.

It follows that one must consider how the addition of calcium chloride to citrated plasma restores coagulation. According to the old and generally accepted concept, it merely furnished calcium ions in excess to the ones depressed by the citrate. On the basis of evidence that prothrombin combines with the

TABLE IV

The Prothrombin Time Obtained on Citrated Plasma by Substituting Magnesium and Strontium Chloride for Calcium Chloride*

Molarity of sodium citrate (1 volume added to 9 volumes of blood)		Prothrombin time				
		0.2	0.15	0.1	0.075	0.05
		sec.	*sec.*	*sec.*	*sec.*	*sec.*
$CaCl_2$	0.01 M	45	15	11	11	11
	0.02 M	11	11	12	12	12
$MgCl_2$	0.01 M	—	180	30	14	14
	0.02 M	120	32	15	14	13½
	0.03 M	36	22	16	16	17
$SrCl_2$	0.01 M	—	60	25	16	14
	0.02 M	58	25	16	16	14½
	0.03 M	26	22	17	18	16

* The citrated blood was kept in ice bath until tested.

citrate radical, a new explanation can be offered; namely, calcium chloride removes the citrate from prothrombin thereby restoring it to its original state. This can be expressed as follows:

$$\text{Prothrombin-citrate} + CaCl_2 \leftrightarrows \text{prothrombin} + \text{Ca citrate complex.}$$

To be sure, the Ca^{++} also are increased.

If this concept is correct, magnesium and strontium should also restore coagulation when added to citrated plasma. The results given in Table IV show that both possess the power to do this. Strontium itself has a much weaker coagulation action than calcium while magnesium has only a feeble activity as Stefanini and Quick (20) have recently shown. Their action can therefore be explained either as liberating prothrombin from the citrate radical or increasing the Ca^{++} by coming in equilibrium with calcium citrate. The equations are:

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(1) Prothrombin-citrate + $MgCl_2$ ⇆ prothrombin + Mg citrate complex.

(2) Ca citrate + $MgCl_2$ ⇆ Mg citrate + $CaCl_2$
$\downarrow$
Ca^{++}.

The fact that strontium and magnesium become less effective as the citrate concentration is increased is in the favor of the first explanation. The greater the concentration of citrate the firmer its union with prothrombin and the less complete the liberation of prothrombin. Were the action of magnesium chloride merely one of increasing Ca^{++}, slight changes in the citrate concentration should be without any appreciable effect on the coagulation time.

Thus the restoration of coagulation in citrated plasma by the addition of calcium chloride can be explained as satisfactorily by the removal of citrate from prothrombin as by increasing the Ca^{++}. Nevertheless, a definitive solution will require additional data obtained by more direct experimental approaches.

The Relation of the Stability of the Labile Factor to the Calcium Concentration of the Plasma

When oxalated human plasma is stored in an open container at ordinary refrigerator temperature, prothrombin activity diminishes relatively rapidly whereas undecalcified or native plasma (such as hemophilic which remains fluid) fails to show this loss (13, 20). Likewise, plasma from blood mixed with 0.1 M sodium citrate (1 volume to 9 volumes of blood) does not show this rapid and marked increase in prothrombin time when stored, but if the concentration of sodium citrate is increased to 0.2 M the disappearance of prothrombin activity is similar to that occurring in oxalated plasma.

In Table V the disappearance of prothrombin activity from plasma under various types of conditions is studied. It will be observed that the prothrombin time increases in 24 hours from a normal of $11\frac{1}{2}$ to 12 to 14 to 15 seconds in oxalated, 0.2 M citrated and amberlite-treated plasma, whereas a decrease or an insignificant increase occurs in the 0.1 M citrated and native plasma. In two plasmas (oxalated and amberlite-treated) in which diminution of prothrombin activity occurs, one is certain that decalcification has taken place. In the native plasma in which the calcium is unchanged, the prothrombin remains essentially unaltered. This clearly suggests that the labile factor, which is responsible for the decrease of prothrombin activity, is stable in the presence of calcium, and is inactivated or destroyed in decalcified plasma. Whether the labile factor itself is a calcium compound cannot be decided until more experimental information is available.

The important suggestion accruing from the observation of the difference in effect of 0.1 M and 0.2 M sodium citrate is that the latter depresses the calcium

ion concentration sufficiently so that the labile factor diminishes, while the weaker concentration of sodium citrate does not. When 0.1 sodium citrate is added to amberlite-treated plasma which is already deprived of calcium, the rate of prothrombin diminution is little affected. This clearly indicates that the stability of the labile factor in 0.1 M sodium citrate plasma is due to insufficient decalcification. In other words, sufficient calcium remains to protect the labile factor. Since even a lower concentration than 0.1 M is enough to inhibit the coagulation of human plasma, an appreciable Ca^{++} concentration must still be present at the citrate level at which incoagulability occurs.

TABLE V

The Influence of the Anticoagulant on the Loss of Prothrombin Activity during Storage

Anticoagulant	Volume used	Volume of blood	Prothrombin time		
			Immediate	After 24 hrs.	After 48 hrs.
	cc.	*cc.*	*sec.*	*sec.*	*sec.*
Sodium oxalate 0.1 M	0.3	2.7	12	15	17½
Sodium citrate 0.1 M	0.3	2.7	11½	10	11
Sodium citrate 0.2 M	0.3	2.7	12	14	15
Amberlite IR-100	—	3.0	11½	14	15
Amberlite IR-100 + sodium oxalate 0.1 M*	0.3	2.7	11½	14	18
Amberlite IR-100 + sodium citrate 0.1 M*	0.3	2.7	11½	14½	18
None‡	—	3.0	12	12½	13

* The sodium oxalate or citrate was added to blood which was previously treated with amberlite IR-100.

‡ By collecting blood in silicone-coated glassware and centrifuging it at high speed, plasma was obtained which remained fluid in silicone-coated test tubes.

Two important facts: (1) the indispensability of the labile factor in coagulation and (2) its instability in decalcified plasma should ultimately serve as important keys to the rôle of calcium in the formation of thrombin.

DISCUSSION

The almost universally accepted idea that it is the ionized calcium of the blood that participates in the coagulation reaction and that it functions as a catalyst is a theory and by no means an established fact. In this study a series of experiments were carried out which yielded results that are not in accordance with this theory. The action of sodium oxalate in inhibiting coagulation is relatively slow. Since the precipitation of Ca^{++} by soluble oxalates is a rapid reaction, the behavior of sodium oxalate cannot be adequately explained by its action of merely removing free calcium. It is more likely that it decalcifies a compound which is essential for the formation of thrombin.

Of particular importance is the discovery that the prothrombin fraction

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which is adsorbed by tricalcium phosphate from oxalated plasma is not removed by this agent from citrated plasma. It appears fairly certain that the citrate radical combines with prothrombin and it is suggested that the prothrombin is thereby inactivated. While this is admittedly not unequivocally proved, it does challenge the explanation that sodium citrate exerts its anticoagulant action by depressing the ionization of calcium. A study of the stability of the labile factor of the prothrombin complex indicates that decalcification is not complete at the citrate level required to stop coagulation completely.

The theory that ionized calcium is required for the activation of prothrombin has been sterile as an aid to unravel the riddle of coagulation and probably has actually led many investigators to treat calcium indifferently. With the evidence marshalled against the theory of the indispensability of ionized calcium, the burden of proof now rests on those who maintain the validity of the theory and it is for them to provide data to support it. This statement has no polemic intent. It is merely to stress the urgency of further study.

The finding that adsorbents such as tricalcium phosphate do not adsorb the prothrombin fraction (which has generally been regarded as the classical unitary prothrombin) from citrated plasma makes the interpretation of several recent studies exceedingly difficult. Lenggenhager's hypothesis (6) of the existence of a prothrombokinin, which is presumably similar to a prothromboplastin, is based on work done on citrated plasma and adsorption with tricalcium phosphate. Recently Milstone (7) in an extended study similar to Lenggenhager's, prepared a fraction which he designates prothrombokinase from euglobulin obtained from citrated plasma. He treated a solution of the euglobulin with $BaSO_4$, apparently to free it from prothrombin. In the light of the present findings, it seems highly probable that his preparation contained some of the prothrombin citrate which is non-adsorbable. To be certain the experiment should be repeated using euglobulin isolated from oxalated plasma. These statements should not be construed as critical evaluations of Lenggenhager's and Milstone's work. They are cited merely to point out the possible pitfalls in applying methods such as adsorption which yield successful results with oxalated plasma, but not with citrated plasma.

The influence of storage on prothrombin activity is another point in question. In oxalated blood a decrease of 50 per cent may occur in 24 hours. Yet Warner, DeGowin, and Seegers (25) found that the prothrombin during storage in citrated plasma decreased only gradually, and required 3 weeks to reach the 50 per cent level. The results recorded in Table V hold the answer to this discrepancy of results. In plasma containing 0.2 to 0.3 per cent sodium citrate, the amount used in blood for transfusion, the labile factor is stable and therefore no rapid decrease in prothrombin activity occurs, in contrast to the rapid fall in oxalated plasma.

SUMMARY

1. The widely accepted theory that calcium participates in the coagulation mechanism in the form of Ca^{++} and acts as a catalyst is not in accord with several important experimental findings:

(*a*) The anticoagulant action of sodium oxalate is much slower than the precipitation of ionized calcium as the oxalate salt.

(*b*) Sodium citrate begins to depress prothrombin activity at a concentration at which ionized calcium is still present. The inability of tricalcium phosphate to adsorb prothrombin from citrated plasma indicates that citrate forms a complex with prothrombin and it is postulated that prothrombin is thereby inactivated.

(*c*) In plasma which is decalcified, *i.e.* in which the Ca^{++} is markedly reduced, the labile factor of prothrombin rapidly decreases. A concentration of 0.01 M sodium citrate sufficient to inhibit coagulation does not depress Ca^{++} enough to cause diminution of the labile factor, whereas when the concentration is increased to 0.02 M the labile factor decreases as rapidly as in oxalated plasma.

2. It is postulated that calcium functions in coagulation not as Ca^{++} but as combined with a component which is part of the prothrombin complex that is not adsorbed by tricalcium phosphate. A concentration of sodium citrate just sufficient to inhibit coagulation is not enough to remove calcium from the essential prothrombin component. The primary anticoagulant action of sodium citrate is therefore not decalcification but antiprothrombic.

3. It has been shown that citrated plasma is basically different from oxalated plasma in several important aspects. Unless cognizance is taken of these differences, serious errors and misinterpretations of experimental findings may be made.

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