INACTIVATION OF ENDOTOXIN BY A HUMORAL COMPONENT

III. Role of Divalent Cation and a Dialyzable Component

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In a preliminary communication (1), we reported that human serum inactivated endotoxin derived from bacterial, mammalian and plant sources but that serum of rabbits and mice did not possess this property. In the accompanying report (2) these observations were extended to the examination of the sera from nine mammalian species. In each of these species the relative effectiveness of citrated plasma and serum in the inactivation of endotoxin was compared. As regards this property of blood the comparison consistently showed that serum was either of low activity or inactive whereas citrated plasma was uniformly more potent. The nature of this striking difference between serum and plasma has been investigated. The various experiments reported here ultimately led to the understanding that the presence in serum of calcium, which suppresses the activity of this endotoxin detoxifying component (EDC), can account for this difference, and that the enhanced effectiveness of plasma in the inactivation of endotoxin is dependent on the binding or removal of calcium ion.

Materials and Methods

The materials and methods employed in this work do not significantly differ from those given in detail in the accompanying paper (2) as regards the following: the collection and processing of blood; the source and dose of endotoxin; the procedure for conducting the ***in vitro*** interaction between endotoxin and the various blood specimens being tested for their EDC activity; the assay for unaltered residual endotoxin in the reaction mixtures as measured by induced hemorrhagic necrosis in mouse tumors.

EXPERIMENTAL

The finding that most sera differed markedly from citrated plasma in EDC activity was consistent in the various species in which they were tested. In an attempt to explain this difference, citrated plasma was clotted by recalcification. The clot which formed was removed and this recalcified plasma was assayed for EDC activity. It was found to have none. The possibility was then considered

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that this difference between serum and plasma was related to clot formation. It was thought that EDC might be adsorbed to the fibrin clot. However attempts to elute the activity from the clot were totally unsuccessful. Other possible explanations were therefore investigated such as the consumption of this activity in the early stages of the clotting process, and of its identity with one of the clotting factors. It was readily demonstrated that the formation of thromboplastin and the conversion of prothrombin to thrombin were not associated with EDC activity. However in the course of these experiments it became apparent that the addition of calcium to plasma resulted in the loss of EDC activity, even when clot formation was prevented by the addition of

TABLE I

Suppression of EDC Activity of Plasma by Addition of Divalent Cations

Human plasma*	Amount	Divalent cation	Amount added	Endotoxin	Induced tumor damage‡	Inactivation of endotoxin
	ml.					
Resin-treated and defibrinted with bovine thrombin	0.04	Ca^{++}	1.1×10^{-2} M	10 µg. *Serr. marcescens*	8/9	None
		Mg^{++}			10/10	"
		Mn^{++}			7/9	"
		Ba^{++}			9/9	"
		None			1/10	Complete

* This plasma contained 2.2 mg. per cent of calcium or 0.06×10^{-2} M.

‡ No. of mice with damaged tumors/total No. of mice tested.

heparin. Furthermore, when blood was collected in heparin, the plasma thus obtained was no different from serum in its EDC activity. Findings such as these showed clearly that clotting *per se* was not implicated in the observed difference in EDC activity between serum and plasma.

Effect of Divalent Cations on EDC Activity.—From the preceding observations it eventually became evident that the loss of EDC which followed recalcification of citrated plasma was independent of clot formation and was due solely to suppression of EDC activity by calcium. This led to an examination of the influence of this and other divalent cations upon the inactivation of endotoxin by serum and plasma.

In order to define better the role of divalent cations in this system it was necessary to avoid the complication posed by the clotting of plasma. To this end, citrated plasma was treated with bovine thrombin to remove its fibrinogen content. Following removal of the clot, this thrombin-treated plasma retained undiminished EDC activity. Addition of the amount of calcium previously employed to recalcify citrated plasma (0.025 ml. M $CaCl_2$/ml. plasma) resulted in total suppression of EDC activity. Table I shows that this effect of calcium

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was duplicated with analogous concentrations of $MgCl_2$, $MnCl_2$, and $BaCl_2$. The divalent cations of cobalt, copper, and iron did not inhibit EDC.

To determine the amount of calcium required for this inhibitory effect it was desirable to obtain plasma to which calcium-binding agents, such as sodium citrate, had not been added. In order to obtain such a preparation, blood from human donors was collected in a Fenwal cation exchange pack and then passed through the resin a second time. The plasmas were separated from the cellular elements and assayed for EDC activity. These resin-treated plasmas were as

TABLE II

Suppression by Calcium of EDC Activity in Resin-Treated Plasma

Human ion-exchange plasma		Calcium concentration		Endotoxin	Induced tumor damage*	Inactivation of endotoxin
Donor	Amount	Amount added	Total amount			
	ml.	*mg. per cent*	*mg. per cent*			
No. 1, contained 3 mg. per cent calcium	0.03	—	3	10 μg. *Serr. marcescens*	0/9	Complete
		2	5		0/9	"
		3	6		4/9	Partial
		4	7		9/9	None
No. 2, contained 2.2 mg. per cent calcium	0.03	—	2.2	10 μg. *Serr. marcescens*	0/9	Complete
		3	5.2		0/10	"
		4	6.2		7/9	None

* No. of mice with damaged tumors/total No. of mice tested.

potent as citrated plasma in the inactivation of endotoxin; the total calcium content of plasma from two donors was 2.2 and 3 mg. per cent as determined by flame spectrophotometry. As is shown in Table II, 0.03 ml. of these plasmas sufficed to inactivate the test dose of endotoxin. Graded amounts of calcium chloride were added to aliquots of each of these samples prior to their incubation with the standard dose of endotoxin. When the total calcium content of the plasma (*i.e.* residual + added calcium) was brought above 6 mg. per cent its EDC activity was completely suppressed; at this calcium level, clotting occurred. These tests were therefore repeated with resin-treated plasma from which fibrinogen had been removed with bovine thrombin. Here, too, in the absence of clot formation, the total calcium level required for the suppression of EDC activity was 6 mg. per cent. Mg^{++}, Mn^{++}, and Ba^{++}, the other divalent cations which had previously been shown to suppress EDC also interfered with the activity of this preparation.

Effect of Anticoagulants.—When it was established that the addition of calcium to plasma resulted in the suppression of EDC activity it was thought

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that the generally low potency of serum might be due to its greater calcium content. If this were the case, it would be anticipated that the addition to serum of calcium-binding anticoagulants would cause the serum to become as effective as plasma in the inactivation of endotoxin. This indeed proved to be the case. The effectiveness of anticoagulants was determined in experiments which utilized rabbit serum lacking measurable EDC activity and human serum of low potency. As seen in Table III, the quantities of these sera em-

TABLE III

Effect of Calcium-Binding Agents on EDC Activity

Reaction mixture					Induced tumor damage*	Inactivation of endotoxin
Blood preparation	Amount	Calcium-binding agent	Concentration $\times 10^{-2}$ M	Endotoxin		
	ml.					
Rabbit serum	0.1	None	—	10 μg. *Serr. marcescens*	7/9	None
		Sodium citrate	2		0/9	Complete
		" oxalate	1		0/9	"
		" fluoride	1.6		0/9	"
		EDTA	0.3		0/9	"
Human serum	0.2	None	—	10 μg. *Serr. marcescens*	7/9	None
		Sodium citrate	2		0/10	Complete
		" oxalate	1		0/9	"
		EDTA	0.3		1/9	"
Human ion exchange plasma, dialyzed	0.03	None	—	10 μg. *Serr. marcescens*	7/10	None
		Sodium oxalate	1		0/10	Complete
		" fluoride	1.6		1/9	"
		EDTA	0.3		0/10	"

* No. of mice with damaged tumors/total No. of mice tested.

ployed failed to affect the tumor-damaging potency of the standard dose of endotoxin. However, the addition of sodium citrate, sodium oxalate, sodium fluoride, or EDTA, resulted in a product which, in its EDC potency, was comparable to the homologous plasma. The amounts of each of these anticoagulants needed to achieve this effect were the same as those required to prevent coagulation of whole blood. Lesser amounts of these agents did not restore EDC activity to serum. In contrast to the pronounced effects of the aforementioned agents, heparin, an anticoagulant which does not bind calcium, is wholly without effect on EDC activity; *i.e.*, heparinized plasma and serum exhibit similar potencies.

Effect of Dialysis of Plasma.—In the initial phases of determining the inhibitory effect of calcium on EDC, attempts were made to remove calcium

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from citrated plasma by dialysis for 24 to 48 hours against 100 volumes of distilled water, at 4°C. The euglobulins, which precipitated, contained no EDC activity and therefore were discarded. The activity was present in the supernatant fluid but the potency was now markedly diminished. For example, where 0.01 ml. of human plasma sufficed to inactivate the standard dose of endotoxin before dialysis, after this treatment 0.2 to 0.4 ml. was required for comparable effect (see Table IV). The dialyzed plasma was found to contain less than 1 mg. per cent of total calcium by flame spectrophotometry. It was therefore anticipated that only 0.1 the amount of oxalate or citrate now would be required to fully restore the EDC activity. Contrary to expectations this amount of oxalate or citrate failed to increase the potency of the dialyzed plasma. However the EDC activity was restored completely when the amount of oxalate or citrate was increased tenfold; this is the amount required to render serum active. These observations were duplicated with sodium fluoride and EDTA. It was thus reasoned that the effect of these anticoagulants in the endotoxin-inactivation system was not limited to their capacity to bind calcium.

TABLE IV

Effect of Dialysis on EDC Activity of Human Plasma

Human plasma	Before dialysis		After dialysis	
	Vol. of plasma reacted with 10 μg. *Serr. marcescens* endotoxin	Induced tumor damage*	Vol. of plasma reacted with 10 μg. *Serr. marcescens* endotoxin	Induced tumor damage*
	ml.		*ml.*	
Ion exchange (donor No. 1).......	0.03	0/10	0.03	9/10
Ion exchange (donor No. 2).......	0.03	0/10	0.03	8/9
ACD-pooled....................	0.01	0/10	0.01	7/9

* No. of mice with damaged tumors/total No. of mice tested.

Evidence for a Dialyzable Component.—The preceding section described experiments in which dialysis of citrated plasma greatly reduced EDC potency. It was found that the activity could be restored upon the addition of its own dialysate. Since the plasma contained added citrate it was at first considered that the citrate present in the dialysate was responsible for this effect. In order to determine whether this effect was indeed due to citrate, it was necessary to obtain dialyzate from plasma to which no anticoagulant had been added. Thus a potent resin-treated plasma was dialyzed against 10 volumes of distilled water for 24 hours at 4°C. The dialysate was lyophilized and taken up to one-half the original volume in water. The dialyzed, resin-treated plasma which had lost appreciable EDC activity (see Table III) was restored to its original potency by the addition of dialysate in an amount equal to that originally present; the dialysate itself did not possess any activity. Thus it is seen that blood contains

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dialyzable material which is required, together with EDC, for the inactivation of endotoxin.

Table V shows that resin-treated, dialyzed human plasma, which no longer exhibited EDC activity at 0.03 ml., had its activity completely restored by the addition of dialysate. Indeed the dialysate still manifested this effect even after it had been boiled for 30 minutes or had been incinerated for 15 minutes. This evidence implies that the component in the dialysate is of inorganic composition.[1]

Effect of EDC in Other Host Reactions to Endotoxin.—The foregoing effects of calcium and calcium-binding anticoagulants on EDC activity were observed with the reaction of tumors in mice as the sole indicator of host response to endotoxin. Preparatory activity for the Shwartzman reaction and lethality for rabbits were used to measure the extent of inactivation of endotoxin by EDC,

TABLE V

Restoration of EDC Activity to Dialyzed Resin-Treated Plasma by a Heat-Stable Substance

Dialyzed plasma		Endotoxin	Induced tumor damage*	Inactivation of endotoxin
Amount	Reconstituted with			
ml.				
0.03	—	10 μg. *Serr. marcescens*	10/10	None
	Dialysate		0/10	Complete
	Boiled dialysate		2/10	"
	Ashed "		0/10	"

* No. of mice with damaged tumors/total No. of mice tested.

in order to ascertain whether the change in endotoxin, as measured by tumor damage, could also be demonstrated using other host reactions as indicator systems.

Shwartzman Reaction.—

Serratia marcescens endotoxin was incubated (40 μg./ml.) for 1 hour at 37°C. with human plasma, recalcified plasma, or with normal saline. Then individual rabbits (New Zealand albinos) were given 0.5 ml. aliquots of each of the three reaction mixtures in the shaved abdominal skin. The provocative intravenous injection of 20 μg. *Salmonella typhosa* endotoxin was given 18 hours later. The hemorrhagic skin reactions which ensued were read 6 to 8 hours thereafter.

The results of one such test are given in Table VI, and show that the preparatory activity of 20 μg. of *Serr. marcescens* endotoxin (*cf.* the controls) was completely eliminated by 0.5 ml. of human plasma. Following recalcification, the plasma no longer affected the preparatory activity of endotoxin.

[1] *Footnote added in proof.*—Physiologic levels of phosphate plus bicarbonate have since been found to restore EDC activity to the same extent as does the dialysate.

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In another experiment the local Shwartzman reaction was used to measure the potency of pooled human plasma in inactivating *Serr. marcescens* endotoxin for comparison with the data obtained by the tumor-damage assay. For this purpose varying amounts of plasma were incubated for 1 hour at 37°C. with *Serr. marcescens* endotoxin so that the aliquots to be injected represented amounts of plasma ranging from 0.1 to 0.003 ml., each reacted with 10 μg. of

TABLE VI

Elimination of the Shwartzman Preparatory Activity of Endotoxin by EDC

EDC		Endotoxin	Skin preparatory activity in rabbits*					
Source	Amount		1	2	3	4	5	6
	ml.							
Human ACD plasma	0.5	20 μg. *Serr. marcescens*	0	0	0	0	0	0
Human recalcified plasma			++++	+++	++	+++	+++	+++
Saline control			+++	++++	++++	++++	+++	++++

* Provoked 18 hours later by intravenous injection of 20 μg. *S. typhosa* endotoxin.

TABLE VII

Titration of EDC in Human Plasma; Inactivation of Shwartzman Preparatory Activity of Endotoxin

Reaction mixture* tested for preparatory activity		Skin preparatory activity in rabbits‡			
Plasma	Endotoxin	A	B	C	D
ml.					
0.1	10 μg. *Serr. marcescens*	0	0	0	0
0.03		0	0	0	Tr
0.01		Tr	0	+++	—
0.005		+++	++++	++++	++++
0.003		++	+++	++++	—
Saline		++	++	++	+++

* Incubated for 1 hour at 37°C.

‡ Provoked 18 hours later by intravenous injection of 20 μg. *S. typhosa* endotoxin.

endotoxin. Following incubation all reaction mixtures were brought up to 0.5 ml. with saline and injected intradermally in the shaved abdominal skin of each of a number of rabbits; 18 hours later the animals were given an intravenous provoking dose of 20 μg. of *S. typhosa* endotoxin and the reactions were read 6 to 8 hours thereafter.

The results of this experiment, given in Table VII, show that, as determined by Shwartzman preparatory activity, the EDC potency of human plasma was similar to that determined by tumor damage. In both test situations the

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minimal amounts of plasma which brought about inactivation of the standard dose of endotoxin was of the order of 0.01 ml.

Lethality.—Among the more common laboratory animals, rabbits are the species most susceptible to the effects of endotoxin. However, individual animals vary considerably as regards the quantity of a potent endotoxin required to produce death. It was shown by Good and Thomas (3) that rabbits pretreated with thorotrast were more susceptible to the lethal effects of endotoxin; *i.e.*, a smaller dose sufficed to kill.

Consequently, in order to use lethality as still another basis for evaluation of changes in endotoxin, New Zealand albino rabbits, weighing 2 to 3 kilograms, were given an intravenous injection of 3 ml. of thorotrast. Six hours later they received, i.v., 20 μg. of typhoid endotoxin which had been incubated in saline, in plasma, or in serum. In the thorotrast-treated animals 20 μg. of typhoid endotoxin represented a uniformly fatal challenge.

TABLE VIII

Elimination of the Lethal Effect of Typhoid Endotoxin by EDC

EDC		Endotoxin	Lethality for thorotrast prepared rabbits
Source*	Amount		
	ml.		
Human serum‡	1	20 μg. *S. typhosa*	4/4
" serum, citrated			0/4
" plasma, resin-treated			0/4
" " " recalcified			4/4
Saline control			4/4

* These products were all derived from a single specimen of blood.

‡ 0.5 ml. of this serum failed to inactivate 10 μg. of *S. marcescens* endotoxin.

From the data given in Table VIII it is seen that resin-treated human plasma eliminated this lethal effect of endotoxin, whereas after it had been recalcified this plasma no longer abolished the lethal property of typhoid endotoxin. On the other hand, serum from the same donor (obtained at the same bleeding), which failed to inactivate endotoxin as measured by the tumor damage assay, likewise failed to prevent death. However, when this same serum was citrated and then incubated with typhoid endotoxin the animals showed no ill effects. Thus the effects of calcium and of calcium-binding agents on the inactivation of endotoxin by EDC are not limited to tumor response but are also evident in other host reactions to endotoxins.

DISCUSSION

The accompanying report described experiments dealing with a humoral component which inactivates endotoxin and whose activity was readily mani-

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fested in citrated plasma whereas little or none was usually apparent in serum. It was pointed out that this marked difference was only discernible with an appropriate bioassay method and with the use of a suitable anticoagulant. The technique used to measure host reactions to endotoxin is of critical importance. The assay method employed in that investigation was considered from the standpoint of its particular suitability for measurement of alterations in endotoxin activity. Only brief mention was made of the crucial role of the anticoagulant used in obtaining the plasma. The data reported in this communication require that further consideration be given to this matter.

It has been shown that EDC activity is inhibited by calcium and certain other divalent cations, *i.e.*, magnesium, manganese, and barium. Thus the high level of activity exhibited by citrated plasma appears to have been achieved by virtue of the calcium-binding property of the anticoagulant. Similar potency was obtained when plasma was derived from blood in which clot formation had been prevented by other calcium-binding anticoagulants, such as oxalate, fluoride, and EDTA, or by the collection of blood through a cation exchange resin. On the basis of these findings it was anticipated that the addition of these calcium-binding agents to serum would disclose EDC activity where little or none was previously evident. This was indeed found to be the case; the addition of these agents to serum rendered it as active as citrated plasma.

While inhibition of EDC activity at first appeared to be a consequence only of calcium concentration, later observations showed that other factors also influenced this activity. For one thing, sera from human donors exhibited considerable differences in EDC potency despite the constancy of calcium levels. Furthermore, in at least one species (rat), serum and citrated plasma were of comparable potency.

Dialysis of plasma against distilled water removed most of the calcium. Contrary to expectation, the EDC potency of the dialyzed material was found to be reduced rather than elevated. Stepwise restoration of calcium, by the addition of graded increments, failed to restore the activity of the dialyzed plasma, excluding the possibility that low levels of calcium were required for EDC activity. On the other hand, addition of "calcium-binding" anticoagulants fully restored activity to dialyzed plasma. Inasmuch as the latter effect was obtained with plasma in which the calcium content had been reduced below the level of detection, it is clear that neither the inhibition of EDC potency with calcium, nor the reversal of this inhibition with calcium-binding anticoagulants is explicable solely on the basis of calcium ion. Obviously, some mechanism other than the binding of calcium is involved.

Since such anticoagulants exerted an important effect even in the absence of detectable calcium, it was considered desirable to obtain plasma without the use of anticoagulant. Accordingly, human blood was passed through a cationic exchange resin to remove most of the calcium; plasma thus obtained proved to be

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as potent as citrated plasma. Dialysis, as in the case of citrated plasma, greatly reduced the potency of the resin-collected plasma. However, when this dialysate was recombined with the dialyzed plasma, EDC activity was fully restored. Moreover, even after the dialysate was dried and ashed, the aqueous extract of the ash restored the potency of the dialyzed plasma to the same extent as did the dialysate itself.

This effect was not attributable to cation for neither divalent (Ca, Mg, Mn, Ba, Cu, Co, Fe) or monovalent (Na, K) cations restored activity. Accordingly, attention is now being focused on the anionic content of the dialysate. At present this much seems likely: some inorganic anionic component, normally present in human plasma, is required for EDC to inactivate endotoxin.

Much of the published work on the effect of plasma upon endotoxic activity was carried out with heparinized plasma. The present study shows that such plasma does not differ from serum in EDC activity. It is only when calcium-binding anticoagulants are employed that plasmas from mammalian species are capable of inactivating endotoxin; moreover, they then manifest a high activity of approximately the same magnitude. This uniformity of effect with plasma from various species is not apparent when serum is tested; pronounced variability in serum is exhibited not only from one species to another but also among individuals within the same species. Appreciation of the critical importance of the animal species used as the source of serum, as well as of the methods employed in obtaining plasma, may warrant re-evaluation of the conflicting statements in the literature on the effects of blood upon endotoxin.

It is still too early a stage in the development of our knowledge of this component to understand its biological implications for the host. It might appear that inactivation of endotoxin could not occur in the intact animal in view of the inhibition of this reaction *in vitro* by levels of calcium such as are known to be present *in vivo*. The fact remains, however, that sera from a number of species exhibit EDC activity despite normal calcium levels. From the limited information now available, it appears that calcium does not directly inhibit the inactivation of endotoxin but rather that it may exert its influence indirectly; *i.e.*, through its effect on some anion which is required for EDC activity.

At present it is not known how the host detoxifies endotoxin. It is now accepted that the cells of the RES take up endotoxin and remove it from the circulation (4). This clearing of endotoxin from the blood does not, however, explain how the toxicity of phagocytized endotoxin is ultimately terminated. The possibility should be considered that EDC contributes to the ultimate detoxification of endotoxin in the circulating blood or within cells.

SUMMARY

The uniformly high potency of citrated plasma as compared with the limited capacity of serum to inactivate endotoxin *in vitro* was found to be a consequence of the anticoagulant employed in collecting the plasma.

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Addition of calcium to plasma suppressed the activity of its endotoxin detoxifying component (EDC) whereas the addition of calcium-binding anticoagulants rendered serum comparable to plasma.

Dialysis of plasma resulted in a marked reduction of its EDC activity despite the concomitant elimination of calcium. EDC activity could then be fully restored upon the addition of calcium-binding anticoagulants.

Resin-treated plasma, without added anticoagulant, had EDC activity equal to plasma obtained with calcium-binding anticoagulants. Following dialysis, resin-treated plasma also sustained a marked reduction in EDC activity which could be fully restored by calcium-binding anticoagulants. Restoration was also obtained with the dialysate even after ashing.

These findings indicated that the suppression of EDC activity by calcium is not direct but is mediated through its effects on an anionic component of plasma which is required for inactivation of endotoxin by EDC.

BIBLIOGRAPHY

1. Landy, M., Skarnes, R. C., Rosen, F. S., Trapani, R-J., and Shear, M. J., Inactivation of biologically active ("endotoxic") polysaccharides by fresh human serum, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96,** 744.
2. Skarnes, R. C., Rosen, F. S., Shear, M. J., and Landy, M., Inactivation of endotoxin by a humoral component. II. Interaction of endotoxin with serum and plasma, *J. Exp. Med.*, 1958, **108,** 685.
3. Good, R. A., and Thomas, L., Studies on the generalized Shwartzman reaction. II. The production of bilateral cortical necrosis of the kidneys by a single injection of bacterial toxin in rabbits previously treated with Thorotrast or trypan blue, *J. Exp. Med.*, 1952, **96,** 625.
4. Thomas, L., The role of the reticulo-endothelial system in the reaction to endotoxins, *in* Physio-pathology of the Reticulo-Endothelial System, (Edited under the direction of B. N. Halpern, B. Benacerraf, J. L. Delafresnaye), Oxford, England, Blackwell Scientific Publications, 1957.

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