more, Kuschke and Gruner's(1) suggestion that reserpine functions as a thyroxine antagonist would lead one to expect that rather than potentiating the toxic effects of thyroid administration reserpine would counteract such effects. In this regard it may be of importance that the dosage of reserpine employed in the present experiment was larger than that employed by others. It is possible, however, that the growth retardation and death obtained when thyroid and reserpine were fed concurrently were due not to a potentiation of thyrotoxicity as a result of reserpine administration but rather a potentiation of reserpine toxicity as a consequence of thyroid feeding.

Summary. Doses of reserpine and desiccated thyroid which were non-lethal to immature rats when administered separately resulted in a 100% mortality within a 2 week period when administered concurrently.

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Effect of Polycarboxylic Acids on Blood Clotting.* (24292)

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The anticoagulant action of citrate has been known for more than 60 years(1). The similarity in molecular structure of isocitrate and aconitate to citrate suggested that these tricarboxylic acids might also interfere with the clotting mechanism. To test this hypothesis, isocitrate, aconitate and the sodium salts of other polycarboxylic acids were incubated with human blood. Of the acids tested, those with 3 free carboxyl groups were effective in prolonging the clotting time.

Methods. Two ml of venous blood from normal volunteers or from patients without known clotting defects was discharged into tubes containing a solution of 0.5 ml of the appropriate test substance and into control tubes charged with 0.5 ml of 0.85% saline. The tubes were stoppered, inverted several times and placed upright in a rack at room temperature. Clotting time was defined as the interval between termination of mixing and formation of a firm clot which remained at the bottom when the tube was completely inverted. The polycarboxylic acids, obtained from commercial sources,† were put into solution, neutralized to pH 7 (pH Hydrion paper) and made up to volume with distilled water or saline just prior to use. The citric acid content of the commercial *cis*-aconitic acid, *trans*-aconitic acid, dl-Na_3 isocitrate, and tricarballylic acid was found to be less than 0.05%

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† Citric acid was purchased from Baker Chemical Co.; *cis*-aconitic acid, dl-malic acid, fumaric acid, tricarballylic acid, malonic acid, dl-isocitric acid lactone and Na_3 isocitrate were purchased from the California Foundation for Biochemical Research, Los Angeles. The source of the other compounds tested was the Nutritional Biochemical Corp., Cleveland, Ohio. *Trans*-aconitic acid, recrystallized from the commercial preparation, was found to be 86% pure by chromatography(2).

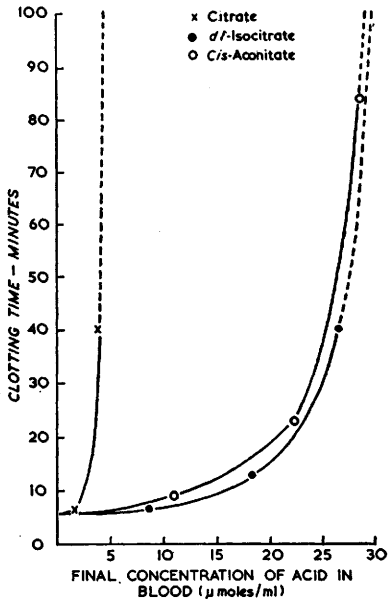

FIG. 1. Effect of varying concentrations of citrate, dl-isocitrate and *cis*-aconitate on clotting time of whole blood. Two ml of blood was added to 0.5 ml of a solution containing the test substance.

as determined by the method of Natelson, Pincus & Lugovoy(3). This amount of citric acid present as a contaminant would not appreciably influence blood clotting time.

Results. Both isocitrate and *cis*-aconitate were found to increase clotting time of whole blood (Fig. 1). The effect was dependent upon the concentration of polycarboxylic acid in the blood. Prolongation of clotting time was identical in the presence of equimolar concentrations of isocitrate and *cis*-aconitate. At a final concentration of 46 μmoles/ml both isocitrate and *cis*-aconitate increased clotting time to over 24 hours (Table I). The unnatural isomer, *trans*-aconitate, prolonged the clotting time, but was less effective than the *cis* isomer. Tricarballylate was also found to influence clot formation. The lactone of isocitric acid was relatively ineffective in increasing the clotting time. Only a slight prolongation of clotting time was noted when each of the dicarboxylic acids, α-ketoglutarate, succinate, fumarate, malate, glutarate, citraconate, glutamate and malonate was added to blood.

In other experiments, 2.0 ml of blood was mixed with 0.5 ml of 0.23 M *cis*-aconitate and permitted to stand for 4 hours. Control tubes contained 0.5 ml of saline and a drop of heparin (1000 U/ml) instead of *cis*-aconitate. At the end of the incubation period the cellular elements were removed by centrifugation, plasma proteins were precipitated with 3 volumes of 20% trichloracetic acid and the citric acid of the supernatant was determined. Plasma that had been incubated with *cis*-aconitate contained 5-7 μg citric acid/ml more than the control. This experiment indicated that a quantity of *cis*-aconitate, insufficient to affect clotting time, had been converted to citrate. Incubation of isocitrate in a similar manner did not reveal conversion of this acid to citrate.

Recalcification times were carried out on plasma from whole blood that had been incubated for 2 hours at room temperature with 0.046 M isocitrate and with *cis*-aconitate. Plasma clots formed between 5 and 10 min-

TABLE I. Effect of Polycarboxylic Acids on Clotting Time of Human Blood. 2 ml of blood mixed with 0.5 ml of 0.23 M acid (neutralized to pH 7). Control tubes contained 2 ml of blood plus 0.5 ml of 0.85% saline. Values are the means of a number (in parentheses) of experiments. Range of values is given beneath mean value in each case.

Acid	Clotting time	
	Control	Sample
Tricarboxylic	Min.	
Citric* (4)	5	>24 hr
Cis-aconitic (4)	5	>24 "
Trans-aconitic (4)	7	5–18 "
Isocitric (4)	6	>24 "
Isocitric lactone (4)	5	30 min.
		13–49 "
Tricarballylic (4)	5	>24 hr
Dicarboxylic	Min.	Min.
α-ketoglutaric (4)	5	10.5
		9–12
Succinic (4)	6	28
		18–41
Fumaric (4)	6	8
		7–9
Malic (7)	5	21
		17–42
Glutaric (4)	6	10.5
		4–15
Citraconic (3)	7	27
		26–29
Glutamic (2)	5.5	10
		8–12
Malonic (5)	5	12
		8–16

* Concentration .115 M.

utes after addition of 0.2 ml of 0.02 M $CaCl_2$ to 0.2 ml of plasma. Clot formation was not apparent 4 hours after $CaCl_2$ had been added to plasma from the tricarballylate incubated blood samples.

Discussion. These experiments indicate that blood clotting is inhibited *in vitro* in the presence of isocitrate and *cis*-aconitate as well as in the presence of citrate. None of the dicarboxylic acids tested was effective in prolonging clotting time; the lactone of isocitric acid was similarly ineffective. The mechanism of the inhibition has not been established. Although other interpretations are possible, it is likely that the tricarboxylic acids chelate with ionic calcium through the 2 terminal carboxyl groups as postulated by Heinz(4) to form relatively undissociable calcium complexes in the blood. Tricarballylate has been shown to complex with ionic calcium in pure solutions(5), but it appears from the recalcification experiments that its ability to prevent clotting stems from interference with the coagulation mechanism other than its Ca^{++} binding ability.

In the normal state it is unlikely that *cis*-aconitate or isocitrate play a significant role in prevention of blood clotting *in vivo* because of the small concentration of these substances in blood(6). It is possible, however, that these intermediates of the Krebs cycle might influence the intracellular concentration of ionized calcium. This concept would be especially important in those tissues, such as nerve and muscle, where small changes in concentration of ionized calcium are known to exert profound effects(7). Conversely, increased concentrations of ionized calcium in tissues might influence citric acid cycle metabolism by complexing with citrate, isocitrate or *cis*-aconitate thereby interfering with enzyme substrate formation.

Summary. Human blood was incubated *in vitro* with the sodium salts of a number of di- and tricarboxylic acids and their effect on clotting time was noted. *Cis*-aconitate, *trans*-aconitate, isocitrate and tricarballylate markedly increased clotting time of whole blood. Prolongation of clotting in the presence of isocitrate and *cis*-aconitate was dependent upon their concentration in blood. The possible mode of action of these metabolically active compounds and the implications of the findings were discussed.

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Role of Relaxin in Stimulating Mammary Gland Growth in Mice.* (24293)

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It has been shown that estrogens (E) will stimulate mammary gland duct growth while E and progesterone (P) in suitable amounts and ratios will stimulate lobule-alveolar growth in normal and gonadectomized male and female animals(1-6). It has been suggested that relaxin synergizes with E and P in stimulating lobule-alveolar growth in rats

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