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Rheological parameters for *Limulus* amoebocyte lysate—endotoxin reaction

Lim K. Hui and William H. Thomas

Department of Pharmacy, University of Otago, Dunedin (New Zealand)

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Summary

The viscosity of coagulin clots, resulting from the reaction between *Limulus* amoebocyte lysate (LAL) and different concentrations of *E. coli* endotoxin, was measured on a rotational viscometer capable of evaluating the shear-rates over a wide range of values. Flow curves were plotted at low to high rates of shear. All the coagulin clots exhibited pseudoplastic behaviour and exhibited irreversible shear breakdown. The Ostwald-de Waele power law equation and the differential form of the constitutive equation were used to describe the pseudoplasticity and the thixotropicity.

Introduction

The amoebocyte lysate of *Limulus polyphemus* forms a gel with minute quantities of endotoxin (Levin and Bang, 1968). The gel is mechanically fragile and will not re-form once disrupted. When increasing concentrations of *E. coli* endotoxin are added to a constant amount of LAL and the reaction allowed to proceed to completion, there is a proportional increase in the formation of the coagulin molecules and network. A relationship between the rate, the extent of coagulin clot formation and endotoxin concentration has previously been reported (Young et al., 1972; Sullivan et al., 1976). Since the viscosity of the coagulin clot is due to the intermolecular network formed, the rheological studies of the clots demonstrate their flow properties and may additionally provide a quantitative assessment of the clot strengths. Flow behaviour studies disclose whether the material follows Newtonian law or exhibits non-Newtonian flow characteristics.

Subjective interpretation, by visual observation, of the clot formation after

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inverting the tube 180°, called the tube test method, does not give a quantitative endpoint. The rheological measurements of clots may provide a better method of detecting the endotoxin reaction as well as offering a means of measuring endotoxin concentration; the rheological studies may also throw light on the nature of the coagulin clot. A change in viscosity is monitored using a rotational viscometer with a plate and cone attachment because of the minute sample sizes (0.1–0.2 ml).

The flow curve is defined by measuring the relationships between shear rate and shear stress or alternatively between flow rate and time. The evaluation of these relationships is mathematically complicated and thus the flow curves are often assessed using mathematical models.

Data presentation

Two equations appropriately describe the flow properties of the coagulin clot, viz. (i) the Ostwald-de Waele equation (Sherman, 1970); and (ii) the differential form of the constitutive equation proposed by Cheng (1973).
The empirical power law of Ostwald-de Waele reads

$$D = \frac{1}{\eta_{app}} \cdot F^{N_s} \tag{1}$$

where D = rate of shear, F is the shear stress, N_s is a constant measure of the internal structure and η_{app} is the 'apparent viscosity', a parameter corresponding to viscosity. It is not feasible, when dealing with non-Newtonian flow to refer to viscosity since it varies with D. For $N_s = 1$, Newton's law applies. The more N_s deviates from unity the more non-Newtonian the flow behaviour.

Pseudoplastic materials follow the Ostwald-de Waele empirical power law equation over a wide range of shear rates. Van de Waarden (1954) suggested that the apparent viscosity η_{app} at any rate of shear has arisen in some degree from the reference viscosity η_r at unit shear rate D.

$$\eta_{app} = \frac{dF}{dD} = \eta_r \cdot D^{N_s - 1} \tag{2}$$

and by integrating this equation with respect to stress dF and rate of shear dD the power law is derived:

$$F = \frac{\eta_r}{N_{s-1}} \cdot D^{N_s} = K \cdot D^{N_s} \tag{3}$$

The log form of Eqn. 2 reads:

$$\log \eta_{app} = \log \eta_r + (N_{s-1}) \log D \tag{4}$$

N_s being an index of the deviation from Newtonian flow behaviour.

In the model proposed by Cheng and Evans (1965), the rheological property is described in terms of scalar constitutive equations consisting of an equation of state and a rate equation. The equation of state is expressed in the form

$$F = \eta(\lambda, D) \tag{5}$$

in which the viscosity, η, is a function of the shear rate, D, as well as the structural parameter, λ, and describes the dynamic shear rate dependency of non-Newtonian flow.

The rate equation reads

$$\frac{d\lambda}{dt} = g(D, \lambda) \tag{7}$$

In the proposed equations the functions, η and g, are subjected to certain restrictions—g is the rate of structural build-up.

Based on these two equations an alternative constitutive relation for thixotropy is described. This takes the form of a differential equation involving two functions, $\alpha(F, D)$ and $\beta(F, D)$. The differential form is obtained by differentiating Eqn. 5 and substituting in Eqn. 6.

$$\frac{dF}{dt} = \alpha(F, D) \cdot \frac{dD}{dt} + \beta(F, D) \tag{7}$$

where $\alpha = (dF/dD)_\lambda$ and $\beta = (dF/d\lambda)_D\, g(\lambda, D)$. α and β are thixotropic functions, α being the function of the material and β the internal structure index and λ a structural parameter. Operating at constant shear rate, β can be obtained from the experimental shear stress versus time relationship. If both F and D are allowed to vary with time, pairs of dF/dt and dD/dt may be obtained for any shear stress and shear rate values, and with β known from a constant shear rate experiment, α can be calculated. However, the β value is more useful since it is directly measurable by experiment.

This report provides an investigation of the flow properties of the coagulin clot and the application of the rheological equations to the flow properties of the clot.

Experimental

Materials

Lyophilized amoebocyte lysate and *E. coli* endotoxin were obtained from two different sources (Associates of Cape Cod and Mallinckrodt Chemical Works, MO). The LAL preparations were reconstituted in a specified quantity of pyrogen-free water as instructed by the supplier. All glassware was thoroughly cleaned and dry-heated at 220°C for 2 h to render apyrogenic.

Sample preparation

Using a micropipette, 0.10 ml of *E. coli* endotoxin solutions of concentration ranging from 0.001 μg/ml to 0.05 μg/ml were added to 0.10 ml of LAL in pyrogen-free test tubes. The mixture was allowed to incubate undisturbed for one hour at 37 ± 0.5°C. Following the incubation, viscosity of the clot formed was measured on a Haake viscometer.

Rheological measurement

Rheological studies were carried out on a rotational cone and plate viscometer (Haake Rotovisko viscometer) using rates of shear from 6.1 to 1000 s^{-1}. The shear stress generated at the cone surface was measured at a constant-temperature of 20 ± 0.5°C. Apparent viscosity measurements were recorded only after a steady state shear stress was attained at all shear rates, which in all cases was reached after approximately 10 s shearing. A hysteresis loop was obtained when the procedure was reversed, that is shear stress measurements at from 1000 to 6.1 s^{-1} rates of shear.

Stress decay studies were carried out at a constant shear rate of 37 s^{-1}. The shear stress was recorded as a function of time at a constant rate of shear.

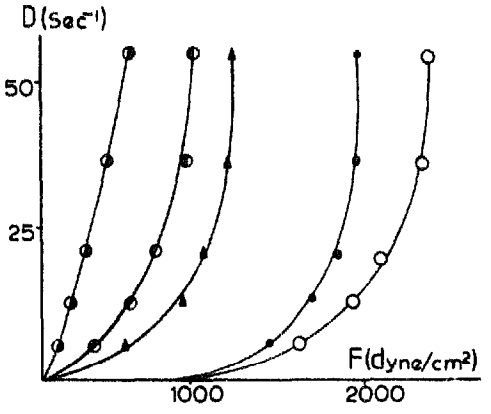
Results and discussion

The coagulin clots show pseudoplastic flow properties under shear. The rheogram (Fig. 1) is a plot of shear stress versus shear rate induced by different levels of *E. coli* endotoxin. All the clots show a rapid decrease in viscosity on increasing the shear rate. The change in shear stress with shear rate is not constant, giving concave flow curves. This is due to a progressive breakdown of structure which is dependent on the degree of agitation as well as being time-dependent.

Fig. 2 is a log–log plot of η_{app} versus shear rate, D, the Ostwald-de Waele power law plot, with slope N_{s-1} (Eqn. 4). These slopes were calculated by the least-squares method. The flow behaviour index, N_s, of each clot is given in Table 1. Two regions of this plot are not in accord with Ostwald-de Waele power law—at the low and at high rates of shear. Many structural dispersed systems show two regions in which the viscosity is independent of shear rate; at very low and very high rates of shear. For such systems a power law can only hold, at best, for that part of the viscosity–rate of shear curve where the viscosity decreases with increasing rate of shear.

The flow behaviour index, N_s, holds only at the part of the curve where the power law applies, suggesting that the shear thinning coagulin clots possess a regular structure when undisturbed; breaking down irreversibly under stress, showing different values of N_s for different gel strengths (D raised to different powers).

Hysteresis loop studies, depicted in Fig. 4 and Fig. 5, also illustrate a progressive breakdown of structure and irreversible thixotropy or rheodestructivity. No regaining of viscosity was observed. The time of shearing is critical as shown; hysteresis loop A results after sample shearing for 10 s at each shearing rate, whilst loop B after sample shearing for 30 s at each shearing rate. Loop area B is smaller than area A illustrating that an increase in shear interval increases structural breakdown (Fig. 4).



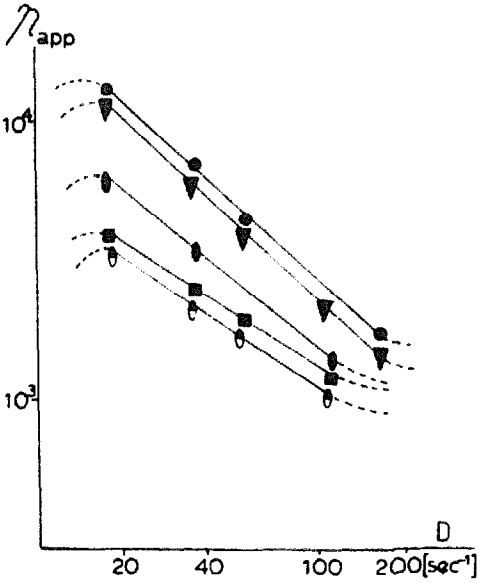


Fig. 1. Flow curves of the coagulin clots exhibiting pseudoplasticity. Key: *E. coli* endotoxin concentration (μg/ml): ○, 0.02; ●, 0.01; ▲, 0.005; ◐, 0.001; ◑, blank.

Fig. 2. Logarithmic–logarimithmic plot of log η_{app} vs log D of the coagulin clots at various rates of shear. Key: *E. coli* endotoxin concentrations (μg/ml): ●, 0.02; ⬮, 0.005; ⬭, 0.001; ▼, 0.01; ■, 0.002.

Fig. 5 illustrates the rheodestructive properties of the coagulin clots. Two hysteresis loops were plotted from results using the same strength test samples and the same shear rate values. The second loop was plotted after the material was left undisturbed for 60 min. One curve does not superimpose on the other, indicating that the structural breakdown is irreversible.

TABLE 1

FLOW BEHAVIOUR INDEX (N_s) OF COAGULIN CLOTS

E. coli endotoxin (μg/ml)	N_s
0.050	−0.0098
0.020	+0.0370
0.015	0.0281
0.010	0.0430
0.008	0.0490
0.005	0.1483
0.002	0.2300
0.001	0.2640
Blank	0.6530

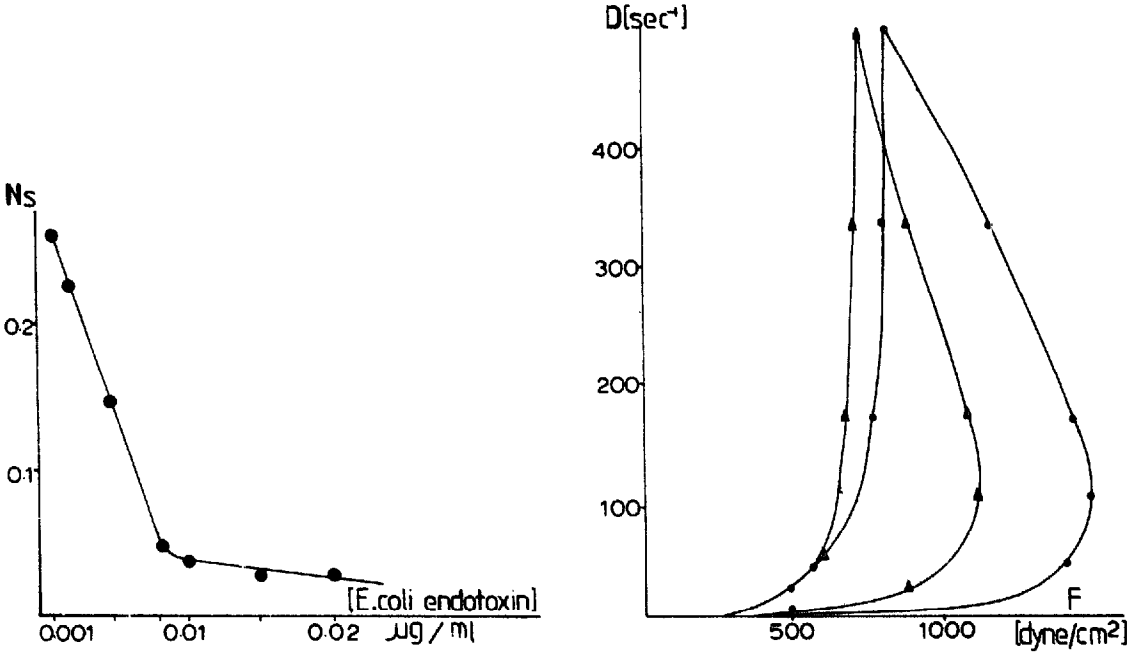


Fig. 3. Plot of N_s, the flow behaviour index of the coagulin clots vs concentrations of *E. coli* endotoxin.

Fig. 4. Effect of shear interval on the 'thixotropic' flow curve of coagulin clot induced by 0.03 µg/ml of *E. coli* endotoxin. Key: ●, shear interval of 10 s; ▲, shear interval of 30 s.

Fig. 6, showing stress decay curves of the coagulin clots sheared at a constant shear rate ($D = 37.04\ s^{-1}$), illustrates that the shear stresses decrease as a function of time and all the curves follow exponential decay process patterns before rheodestruction occurs as indicated by the log plots (Fig. 7).

From the stress decay measurements of the clots, β, the internal structure index, can be obtained by plotting $(F_t - F_\infty)$ versus time. It is the gradient of the semi-log plot (Fig. 7), calculated by the least-squares method, and is related to the differential form of the constitutive equation. All the β values are negative and therefore the modules bracket, $|1/\beta|$, is used to avoid taking roots of negative quantities—Table 2. Fig. 8 shows the plot of $|1/\beta|$ versus *E. coli* endotoxin concentration. It is apparent that the internal structure index is a function of the strength of the coagulin network. The plateau indicates that maximum polymerization of the coagulin molecules does not bring about a marked difference inthe β value.

The pseudoplasticity and thixotropy or rheodestructivity of the gel suggests the presence of a network structure which is continuously broken down irreversibly under stress. This also explains the spectral change in viscosity and the phenomenon of hysteresis.

In any discussion of mathematical models the implication of any dynamic response of the test material must be considered.

In the power law, attributed to Ostwald and de Waele, N_s, being a measure of the deviation from Newtonian behaviour may be used to indicate the *E. coli* endotoxin

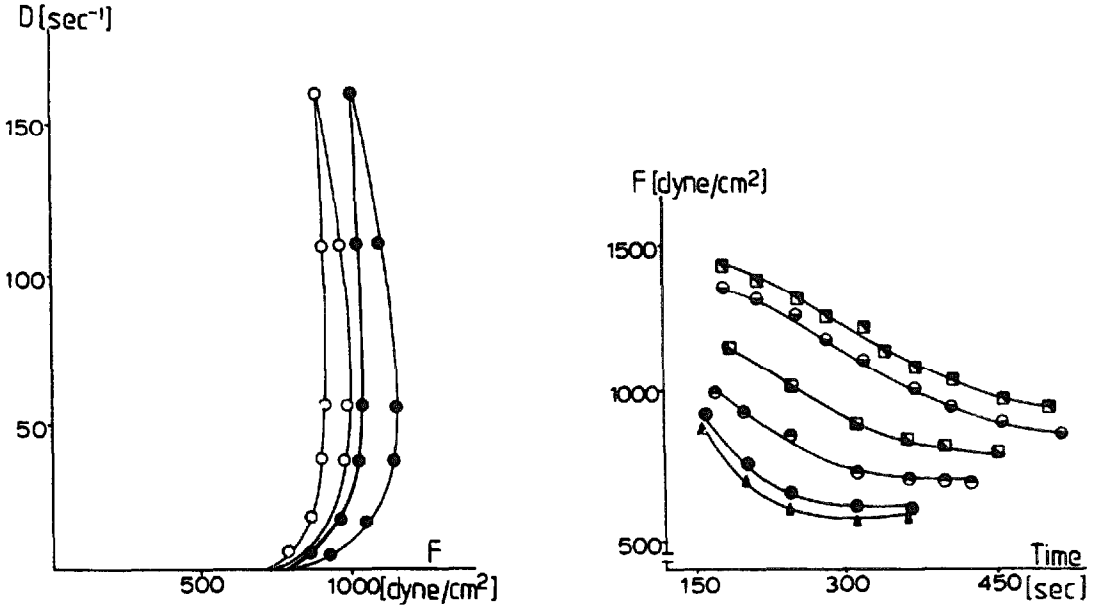


Fig. 5. Recycling hysteresis loop test of the coagulin clot induced by 0.005 μg/ml of *E. coli* endotoxin. The second hysteresis loop (○) was recorded 60 min after the completion of the first shear cycle (●). Shear interval for each shear rate was 10 s.

Fig. 6. Stress decay curves of the coagulin clots shear at constant shear rate, $D = 37.04\ s^{-1}$. Key: concentrations of *E. coli* endotoxin (μg/ml): ◪, 0.05; ◒, 0.03; ◪, 0.01; ◒, 0.005; ●, 0.003; ▲, 0.001.

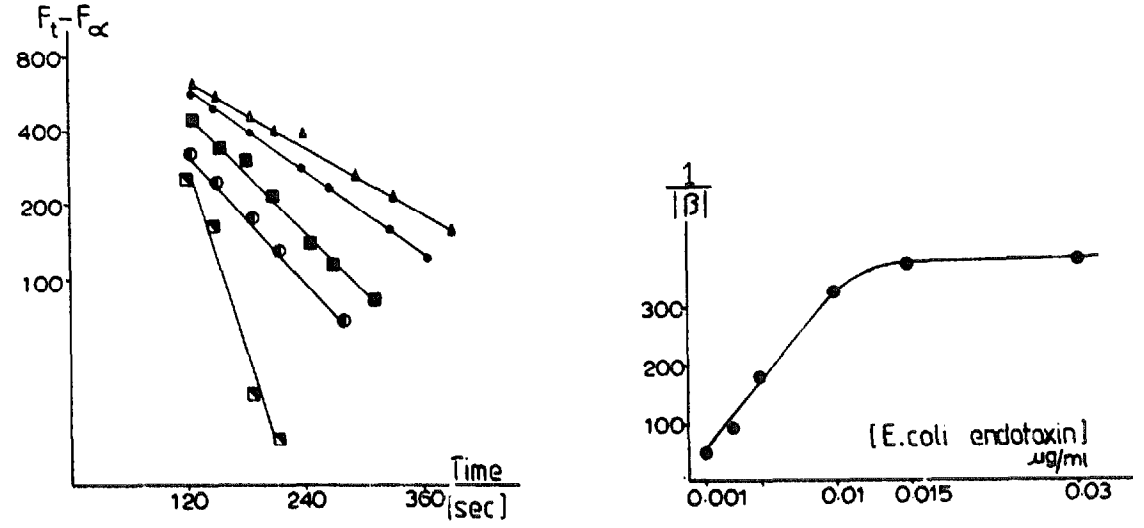


Fig. 7. Rate equation illustrating variation of shear stress with time at constant shear rate for the coagulin clots induced by different levels of *E. coli* endotoxin. Key: concentrations of *E. coli* endotoxin (μg/ml). ▲, 0.05; ●, 0.03; ■, 0.01; ◐, 0.005; ◪, 0.003.

Fig. 8. Plot of $|1/\beta|$ versus concentrations of *E. coli* endotoxin. β is the internal structure index of coagulin clot.

TABLE 2

INTERNAL STRUCTURE INDEX, β, OF COAGULIN CLOTS INDUCED BY DIFFERENT LEVELS OF *E. coli* ENDOTOXIN. $|1/\beta|$ = THE MODULUS BRACKET

E. coli endotoxin (μg/ml)	β	$1/\beta$
0.001	−0.0193	52
0.003	−0.0109	92
0.005	−0.0055	182
0.010	−0.0031	328
0.030	−0.0026	380
0.050	−0.0027	370
Blank	−0.0000	-

concentration necessary to induce the coagulin clot (Fig. 3) since a relationship exists between the N_s values, the pseudoplasticity, the extent of gelation and the endotoxin concentration. These values are obtained over a wide range of viscosity measurements at variable shear rates.

The thixotropicity of the coagulin clot is difficult to study by the hysteresis loop method. The shape and size of these loops depend on the dimension of the cone, the viscosity of the clot and the sweep time of the loop experiment. The gel structure either does not recover or the recovery is too delayed to be detected experimentally. Assessment of the time-dependent clot is desirable at time relative to the degree of structure breakdown at which viscosity measurements are made.

A rheological structural reference point is impossible as important considerations such as the response of the instrument and a knowledge of the shear history of the gel is unavailable. Consequently, the two simplest reference points are at $t = 0$, when the material is at the maximum structural state, and at time $t = \infty$, when maximum structural breakdown occurs. At this point ($t = \infty$) a relatively long period of shearing has elapsed when the shear history of the material is less important and may be ignored. The measurements are thus based on a rate process of structural breakdown, when constant shear rate studies are done which indicate a shear stress exponential decay process, (Fig. 6) the constitutive equation applies. The rate of structural breakdown (rate constant β) reflects the clot strength and thus the endotoxin concentration needed to induce gelation (Fig. 8).

Increased gel formation parallels proportionally increased network formation and endotoxin contamination. Coagulin molecules polymerize into helical secondary structures (Solum, 1973) and are composed of fine fibres of 50–100 μm diameter (Gaffin, 1976). In this rheological study it is assumed that coagulin polymers form a unique network of aggregates composed of helical structures stabilized by permanent dipole attractions and Van der Waals forces. The coagulin polymers wind or overlap in opposite polar direction to reach a steady-state. Under shear, the overlapping helices align 'tail-to-tail' causing electrostatic repulsion thus avoiding interparticle

attachment or coagulation. The breakdown of Van der Waals forces is assumed to be caused by activation energy for viscous flow.

The viscosity of the gel resulting from the intermolecular network and its structure is used as a measure of the gel strength and is indirectly proportional to the amount of endotoxin present in the sample.

N_s (flow behaviour index) and β (internal structure index) are quantitative parameters that can be used to measure the endotoxin concentration. Gel formation resulting from the combination of LAL and endotoxin or pyrogen-contaminated solutions affords a simple specific and rapid test. Rheological studies provide a quantitative assessment of bacterial endotoxin contamination.

References

Cheng, D.C.-H., A differential form of constitutive relation for thixotropy. Rheol. Acta, 12 (1973) 228–233.

Cheng, D.C.-H. and Evans, F., Phenomenological characterization of the rheological behaviour of inelastic reversible thixotropic and anti-thixotropic fluids. Br. J. Appl. Phys., 16 (1965) 1599–1617.

Gaffin, S.L., The clotting of the lyzed white cells of *Limulus* induced by endotoxin I: Preparation and characterization of clot-forming proteins. Biorheology, 13 (1976) 273–280.

Levin, J. and Bang, F.B., Clottable protein in *Limulus*; its localization and kinetics of its coagulation by endotoxins. Thromb. Diath. Haemorrh., 19 (1968) 186–197.

Sherman, P., Industrial Rheology, Academic Press, London and New York, 1970, p. 98.

Solum, N.O., The coagulogen of *Limulus polyphemus* haemocytes: a comparison of the clotted and the non-clotted form of the molecule. Thrombosis Res., 2 (1973) 55–61.

Sullivan, J.D., Valois, F.W.M. and Watson, S.W., Mechanisms in Bacterial Toxicology, John Wiley, New York, 1976, p. 217.

Van de Waarden., Viscosity and electroviscous effects of emulsions. J. Colloid Sci., 9 (1954) 215–222.

Young, N.S., Levin, J. and Prendergast, R.A., An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. J. Clin. Invest., 51 (1972) 1790–1797.