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The anticoagulant activity of heparin: measurement and relationship to chemical structure*

T. W. BARROWCLIFFE,† B. MULLOY, E. A. JOHNSON and D. P. THOMAS

National Institute for Biological Standards and Control London, UK

Abstract: For many years the anticoagulant activity of heparin has been estimated by coagulation assays, in which the prolongation of clotting times by heparin is measured under various conditions. More recently, assays have been developed which measure the inhibitory action of heparin on isolated coagulation enzymes, notably Factor Xa and thrombin, using specific amidolytic peptide substrates.

The anticoagulant activity of heparin arises primarily from its ability to bind to antithrombin III (AT III), altering the conformation and enhancing the activity of this major protease inhibitor. Passage of heparin through an immobilised AT III column yields two fractions: a high affinity fraction with 300–350 iu mg^{-1} anticoagulant activity, comprising one-third of the total, and a low affinity fraction with an activity of less than 10 iu mg^{-1}, comprising the remaining two-thirds. Studies in several laboratories have demonstrated that a specific pentasaccharide sequence is required for AT III binding. The authors have shown that the presence or absence of this sequence can be detected by high-field proton NMR, thus providing a semi-quantitative method for a functionally important group.

A second major influence on anticoagulant activity is molecular weight distribution. Studies in the authors' laboratory on a series of fractions of 5000–35,000 showed that whereas anticoagulant activity in APTT clotting assays decreased with decreasing molecular weight (Mr), activity in anti-Xa assays was maintained or increased in the low Mr fractions. However, *in vivo* studies showed that high affinity fragments with anti-Xa activity only were poor antithrombotic agents. It appears that the presence of the AT III binding site alone is not sufficient for full antithrombotic activity; an extra length of polysaccharide chain of at least 15 residues is required. Molecular weight distribution is readily assessed by HPLC, although the lack of suitable reference materials hampers assignment of absolute molecular weights.

Important determinants of anticoagulant activity can now be assessed by physico-chemical techniques but, at present, these techniques are not precise enough to replace anticoagulant assays as predictors of *in vivo* behaviour.

Keywords: *Heparin assays; heparin structure; LMW heparin.*

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†Address all correspondence to Dr T. W. Barrowcliffe, Division of Haematology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3QG, UK.

Introduction

In this paper, the various methods for the measurement of the anticoagulant activity of heparin are briefly reviewed. The molecular basis for the anticoagulant action of heparin is discussed and some physico-chemical techniques, which are useful in the characterisation of various heparin preparations, are mentioned.

Measurement of anticoagulant activity and relationship to chemical structure

Development of anticoagulant assays

The earliest assays of the anticoagulant activity of heparin utilised whole blood from experimental animals. The unit of activity was first defined by Professor Howell as the amount of heparin which prevented the coagulation of fresh cat's blood when held for 24 h at 0°C [1]. Subsequently, a large number of modifications were introduced by various groups of workers and these are reviewed in detail elsewhere [2–4]. The main variations in anticoagulant assays have involved: different species of blood (e.g. cat, dog, chicken, human); use of whole blood or anticoagulated plasma; type of anticoagulant; use or non-use of accelerators (e.g. tissue thromboplastin); use of thrombin; and type of measurement (degree of coagulation or coagulation time).

All these various methods, however, can be classified as *in vitro* bioassays, i.e. they involve the use of isolated blood or plasma other than whole animals.

Pharmacopoeial methods

Because of the profusion of assay methods, standardised methodology was developed by the British and United States Pharmacopoeias. The USP method is an adaption of that of Kuizenga *et al.* [5], in which heparin is added to citrated sheep plasma in the absence of any added accelerators, and the degree of coagulation measured after 1 h. Although the method can be very precise in experienced laboratories, the inter-laboratory variability can be somewhat greater, probably because of differences in the substrate plasma [6].

The BP method, established in 1953 and used until 1983, was that of Adams and Smith [7] and differs in several respects from the USP method. The substrate is whole ox blood, anticoagulated with sodium sulphate; an accelerator, tissue thromboplastin (a crude ox brain extract), is used and the coagulation time, rather than the degree of coagulation, is measured. Thus for 30 years the BP and USP methods differed in almost every major respect.

Since 1983, a new BP method has been adopted, which is virtually identical to that now published in the European Pharmacopoeia (EP). The method uses citrated sheep plasma, kaolin and cephalin and is thus basically an APTT method with sheep plasma; however, unlike the USP, the method of collection of the sheep plasma is precisely specified.

Modern anticoagulant assays with human plasma

A major drawback of all the pharmacopoeial methods is their use of animal blood or plasma. Whereas animal plasma can give similar results to human plasma when test and standard samples are similar [8], this may not be the case when the test and standard heparins differ in tissue of origin [9, 10] or in molecular weight [11]. In the authors' laboratory, heparin samples are characterised by a variety of assay methods based on human plasma:

APTT. This is the most widely used test for monitoring heparin therapy in clinical laboratories and the most reliable general purpose method for measuring the anticoagulant ability of heparin. In an international collaborative study, the method showed good precision, both within and between laboratories, with relative standard deviations (RSD) near 4% [12].

Anti-Xa. Unlike the APTT, this method measures the ability of heparins to inhibit a single enzyme in the coagulation cascade. Two versions of the assay are used, one in which residual enzyme is measured by a clotting assay [13, 14] and the other in which enzyme activity is measured with a specific amidolytic peptide substrate; the enzyme releases the chromophore *para*-nitroaniline, which can be quantified in a spectrophotometer [15]. Intra-laboratory precision is similar with both methods, but variability between laboratories is greater with the clotting method and differences in potencies with the two methods have been found for low molecular weight (LMW) heparins [16].

Thrombin time. Inhibition of the thrombin time by heparin is one of the oldest and simplest methods but is also one of the most difficult to perform precisely. Small variations in assay conditions have marked effects on clotting times and the concentration range for the linear part of the standard curve is very narrow. Since the results of thrombin time assays agree closely with those by APTT, the authors place most reliance on APTT assays in their laboratory. Recently, methods based on inhibition of the amidolytic activity of thrombin towards a small peptide substrate have been described [17]; these methods may offer improved precision over thrombin clotting assays.

These assay methods are reviewed in detail elsewhere [4]. Examples of APTT and anti-Xa assays from the authors' laboratory are shown in Figs 1 and 2.

Biochemical basis of anticoagulant activity

Within the last 15 years, substantial advances have occurred in knowledge of the molecular basis of the anticoagulant activity of heparin and hence of the assay methods described above. The basis of the action of heparin is that it binds to antithrombin III (AT III), the major inhibitor of the coagulation system in plasma. AT III is not only the major inhibitor of thrombin but also inhibits most other coagulation enzymes, i.e. Factors Xa, IXa, XIa and XIIa and, when heparin binds, it induces a conformational change which greatly increases the rate of inhibition of these enzymes by AT III [18]. Although all enzymes are inhibited more rapidly, it now seems likely that the anticoagulant effects *in vitro*, and possibly the antithrombotic action *in vivo*, are predominantly due to increased inhibition of thrombin and Factor Xa.

When heparin is fractionated by its ability to bind to a column of immobilised AT III, it is found that only 30–40% of the molecules bind with high affinity [18]. After elution, this subfraction has high anticoagulant activity (approx. 350 iu mg^{-1}, compared with 160 iu mg^{-1} for unfractionated heparin), whereas the low affinity component, comprising 60–70% of the total, has low anticoagulant activity (<10 iu mg^{-1}). Thus the anticoagulant activity of heparin is accounted for mainly by a subfraction of molecules comprising about one-third of the total.

This major difference in ability to bind to AT III implies some structural differences between the high and low affinity components. Fragmentation of the high affinity material, followed by detailed carbohydrate analysis, has been carried out by Lindahl and co-workers [19] and has revealed the presence of a unique pentasaccharide which

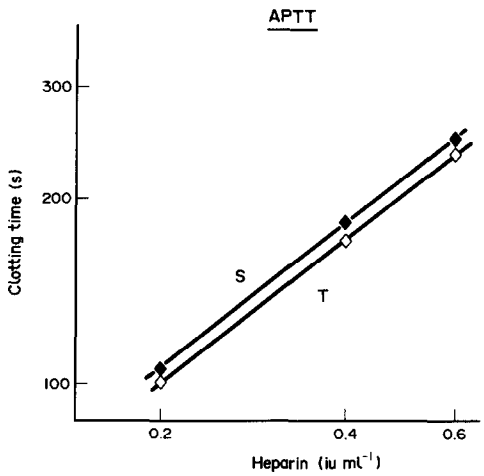

Figure 1
An example of an APTT assay using human plasma. S = fourth IS for heparin; T = commercial heparin (unfractionated).

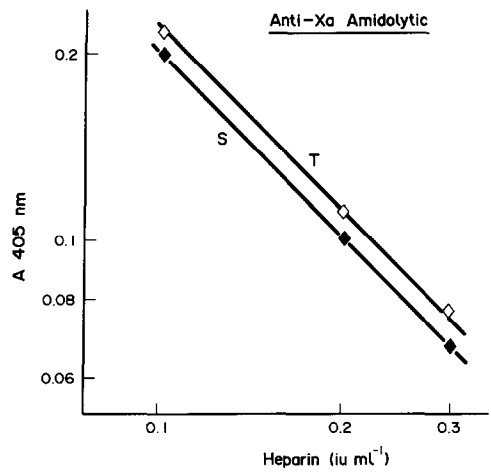

Figure 2
An example of an anti-Xa amidolytic assay using human plasma. S and T are as in Fig. 1.

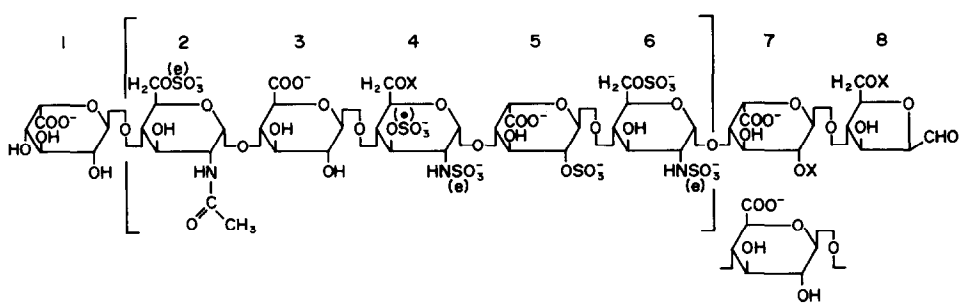

Figure 3
Structure of the antithrombin binding octasaccharide prepared by partial deaminative cleavage of heparin with nitrous acid. The actual binding region is represented by the pentasaccharide sequence (units 2–6) within the brackets. The 3-O sulphate group of unit 4 (marked by an asterisk) is unique to the antithrombin binding region of heparin, while each one of the sulphate groups indicated by (e) is essential to the high affinity binding of antithrombin. Reproduced with permission from Björk and Lindahl [18].

constitutes the actual AT III binding sequence; this is illustrated in Fig. 3. Within this sequence, certain residues are essential for AT III; of particular importance is the O-sulphate group on position 3 of glucosamine residue 4, which occurs only in the AT III binding site and nowhere else.

The structure and activity of this pentasaccharide have now been confirmed by chemical synthesis.

NMR studies on heparin

High-field proton NMR is a technique capable of giving a great deal of structural information about biological macromolecules of all kinds. The spectra of heparin samples are extremely complex but, with the aid of the very high-field NMR spectrophotometers now available, can provide detailed information on the composition, sequence and conformational properties of heparin, all without the need for degradation of the sample.

The spectra are dominated by signals from the two most abundant disaccharide repeating sequences, [→4)-L-IdoA2SO_3^--(1→4)-D-GlcNSO_3^--6SO_3^--(1→] and [→4)-D-GlcA-(1→4)-D-GlcNAc-(1→] [21, 22], and their overall appearance is of a superposition of the spectra of these two in their respective proportions. Other less intense signals arise from less common structures in which substitution with O- and N-sulphate varies from the above patterns. By comparison with the spectra of chemically-modified heparin and heparin sulphates, in which the substitution patterns have been systematically altered, some of the minor signals (most usefully those from the anomeric proton of each monosaccharide residue) have been assigned [23]. These anomeric signals have chemical shifts which are sensitive not only to the structure of the residue itself but also to that of the adjacent residue linked at C1, so providing sequence information.

Comparison of the spectra of fractions of heparin with high and low affinities for immobilised antithrombin reveals that several minor anomeric signals are considerably enhanced in the high affinity sample. Using the sequence-specific assignments from modified heparins and comparison with the published spectrum of a synthetic pentasaccharide with high affinity for antithrombin [24], these signals can be assigned to the individual monosaccharide residues of the naturally occurring high affinity sequence in the polysaccharide (Fig. 4).

In principle, therefore, high-field proton NMR can provide a purely physico-chemical method of determining the content of high antithrombin affinity heparin in any given sample and therefore of predicting its antithrombin-mediated activity. In practice, however, the high cost and low quantitative accuracy of the method render it unsuitable for use on a routine basis.

Influence of tissue source on anticoagulant activity

If binding of AT III were the only determinant of anticoagulant activity, samples of heparin assayed against a standard should give the same results by all assay methods. An early indication that this was not the case came from the results of an international collaborative study; samples of mucosal heparin, when assayed against the second IS (lung heparin), gave significantly different results by the BP and USP methods [6]. A major consequence of this discrepancy was that, upon replacement of the second IS (lung) with the third IS (mucosal), samples of heparin assayed by the USP method against the USP standard differ by some 7% from their potency in International Units. More recent studies, using human plasma based assays, have shown that lung heparins

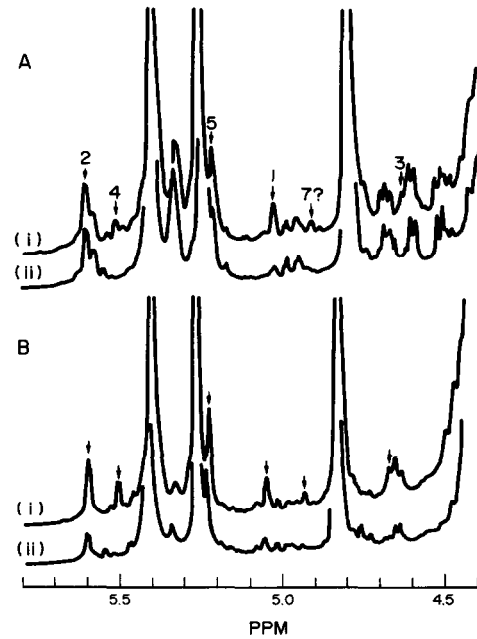

Figure 4
Partial ^{1}H-NMR spectra at 500 MHz (343 K) of heparin fractions porcine of (i) high and (ii) low affinity fractions of heparin, mucosal (A) and bovine lung (B) (adapted from Ref. 23). The arrows indicate the H-1 doublets enhanced in both mucosal and lung high-affinity fractions, numbered to correspond with the monosaccharide units of the antithrombin binding sequence in Fig. 3.

assayed against a mucosal standard have lower potencies by anti-Xa assays than by the APTT method [10]. This difference is apparent across the Mr range and probably reflects lower affinity for AT III in lung heparin, since anti-Xa activity mainly correlate with AT III binding. Activity in APTT assays also involves thrombin binding (see below) and this may occur more readily in lung heparin because of its more regular structure.

Influence of molecular weight on anticoagulant activity

Heparin is highly polydisperse, with a Mr range of 5000–30,000 and a mean of about 12,000–13,000. It has been known for some time that anticoagulant activity, measured by pharmacopoeial or APTT methods, decreases with decreasing molecular weight [23, 24]. However, studies in the authors' laboratory showed that the molecular weight dependency using an anti-Xa assay was completely opposite to that by APTT, with activity increasing towards lower molecular weight [25].

Subsequent studies confirmed these findings and showed that the molecular weight dependence of thrombin inhibition assays was similar to that of the APTT method, i.e. a decrease towards lower molecular weight [10, 26, 27]. Thus assays measuring inhibition of the two major coagulation enzymes, Factor Xa and thrombin, show a completely different molecular weight dependence, implying major differences in the mechanism of the anticoagulant activity of heparin against these two enzymes. The main reason for these differences is that inhibition of thrombin requires the heparin chain to be able to bind to both thrombin and AT III; with lower molecular weight, there is decreased probability of the chain being long enough to accommodate both proteins, especially since the AT III binding sequence may be located anywhere in the chain. Below a certain size, about 16–18 saccharides, the heparin molecules are unable to bind both thrombin and AT III and therefore heparin fragments below this size have no anticoagulant activity against thrombin [28]. For Factor Xa, there is no such requirement for enzyme

binding and therefore anticoagulant activity against Factor Xa is retained down to the smallest saccharide chain able to bind AT III, the pentasaccharide [20]. In a study of the molecular weight dependence of the activity of heparin against various coagulation enzymes, Holmer *et al.* [29] found that Factors IXa and XIa behaved like thrombin, with decreasing activity towards lower molecular weight, whereas Factor XIIa was similar to Factor Xa, with activity being retained at lower molecular weight. Inhibition of Factors IXa and XIa is probably involved in heparin prolongation of the APTT, and this would explain the decreasing activity with decreasing molecular weight in this assay.

Standardisation of low molecular weight heparins

In a collaborative study of eight different LMW heparins, assayed against the fourth IS for unfractionated heparin by APTT and anti-Xa methods in eight different laboratories, several problems were noted [16]. Apart from the differences in potency by different assay methods as previously mentioned, there were large differences in potency between different laboratories performing the same assay method. Whereas inter-laboratory variability, expressed as RSD, was less than 10% for unfractionated heparin [12], RSD values for LMW heparins were mostly over 30%, with potency estimates varying over a three-fold range [16]. In addition, in most of the assays, dose response lines of LMW heparins were not parallel to those of the standard.

It became clear from the results of this study that unfractionated heparin was an unsuitable standard for measurement of the anticoagulant activities of LMW heparins. However, when one of the eight LMW heparins was selected as an arbitrary standard for measurement of the other seven preparations, parallel line assays were obtained and the inter-laboratory variability was much reduced (Table 1). Accordingly, it was decided to proceed with the establishment of a separate international standard for LMW heparin. In view of the differences between the various LMW heparins being manufactured, selection of material for a standard had to be made carefully. Three candidate preparations were chosen as being in the "middle of the spectrum" with respect to molecular weight distribution and anticoagulant activities. These three preparations, together with the International Standard for unfractionated heparin, were subjected to collaborative study in 25 laboratories using six different assay methods. Following the results of this study, one of the preparations (in ampoules coded 85/600) was established by WHO as the first IS for LMW heparin [30] and it has been recommended by the Heparin Subcommittee of the International Committee on Thrombosis and Haemostasis

Table 1
Inter-laboratory variability* of anti-Xa assays of LMW heparins: comparison of unfractionated and LMW heparin standards

LMW heparin test sample	RSD %		
	UFH standard	LMWH standard F	LMWH standard G
B	49.5	9.9	14.1
C	23.3	13.7	8.6
D	43.4	11.1	12.5
E	49.7	11.7	14.1
F	36.3	—	6.4
G	32.6	6.4	—
H	30.4	4.4	5.6
I	34.8	4.9	5.0

* Data from international collaborative study of LMW heparins, NIBSC report (1985).

that all manufacturers calibrate their LMW heparin products against this new standard [31].

Molecular weight analysis of heparin

The measurement of the Mr of highly charged polysaccharides, such as heparin, by orthodox physical methods, e.g. by equilibrium sedimentation, is problematic in practice if not in theory; also, heparin samples are highly polydisperse. With the appearance in clinical use of LMW heparin fractions or fragments, assessment and control of Mr has developed a new significance.

LMW heparins are normally prepared by controlled depolymerisation by any one of a number of different procedures, many of which yield predominantly even-numbered oligosaccharides, the Mr values of which may be estimated with fair accuracy (using a suitable mean Mr value per saccharide) if the number of disaccharide units can be counted. Using high-performance size exclusion chromatography (HPSEC), this can be done at the lower end of the Mr range (Fig. 5) only, and a complex relationship between Mr and K_{av} makes the validity of extrapolation questionable.

Polyacrylamide gel electrophoresis (PAGE) is also capable of resolving the individual even-numbered oligosaccharides. It has a much higher resolution than HPSEC and the relation between Mr and migration rate follows a simple law with some exactitude (Fig. 6), making a degree of extrapolation acceptable. Unfortunately, quantitative densito-

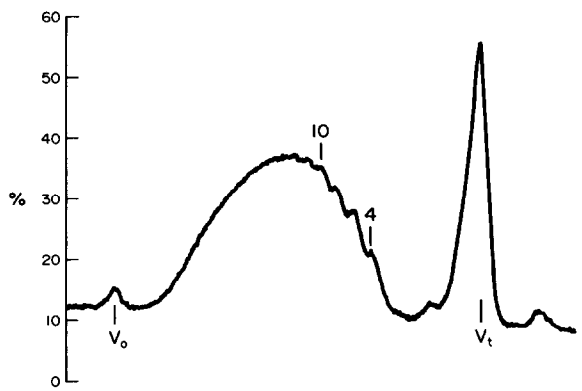

Figure 5
HPSEC of a LMW heparin sample: TSK G2000SW, 0.3 M $NaClO_4$. Tetra- and decasaccharides are indicated.

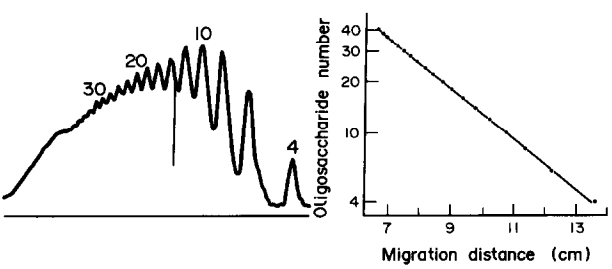

Figure 6
Densitometer trace from PAGE of LMW heparin sample as used for Fig. 1 and relation between migration distance and Mr. Oligosaccharide numbers marked.

metry of LMW heparins separated on PAGE gels is as yet unreliable, but PAGE may nevertheless be used to estimate accurately the mean Mr of narrow-cut fractions (prepared by larger scale SEC) which may then be used as standards for HPSEC. In this way the full Mr range of LMW heparins may be covered in a reproducible manner [32].

The significance of the Mr distribution is demonstrated in Fig. 7, in which the ratios between pairs of results by four different assay procedures are plotted against the percentage of material with Mr > 7500 in seven different LMW heparins. Mr was determined by planimetry of the HPSEC trace. Areas pertaining to material with Mr < 2500 were subtracted as inactive before determining the percentage; their inclusion gave similar plots with slightly lower correlation coefficients. The heparins and assay results are those given (samples C to I only; B is not a heparin) in Table 2 in the paper by Barrowcliffe *et al.* [16]. Since five essentially different preparative procedures have been used by six manufacturers to make these samples, it is reasonable to conclude that different preparative methods in themselves play a secondary part in determining the potency of the product (at least by those assay procedures) when compared with the resulting Mr range.

Conclusions

The anticoagulant activity of heparin can be measured by several *in vitro* methods, of which the APTT and anti-Xa assays using human plasma are the most preferred. Anticoagulant activity requires a specific pentasaccharide sequence which is responsible for binding to AT III; in addition tissue source and Mr are important determinants of anticoagulant activity. Differences between molecules with high and low affinity to AT III can be detected by high-field proton NMR. Molecular weight distribution is an

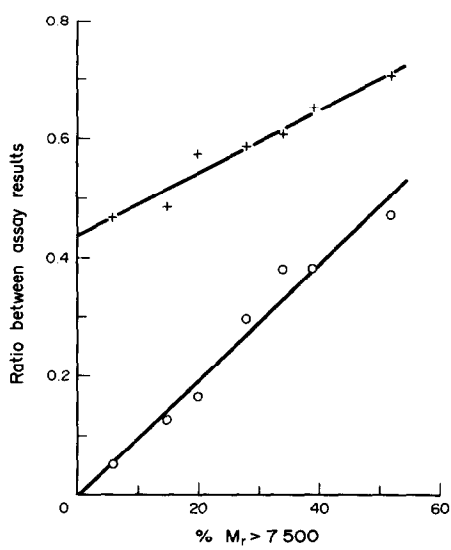

Figure 7
Relationship between assay ratios and higher Mr content of LMW heparins (see text).

○, $\frac{\text{APTT}}{\text{anti-Xa clotting}}$; +, $\frac{\text{anti-Xa, amidolytic, AT III}}{\text{anti-Xa, amidolytic plasma}}$.

important characteristic, especially of LMW heparins, and can be determined by HPSEC or PAGE.

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