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Partial Purification of the Template-Active Fraction of Chromatin: A Preliminary Report

(chromatin fractionation/DNase II/DNA·RNA hybridization)

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ABSTRACT **A fraction of rat-liver chromatin that is transcriptionally active *in vivo* has been purified 6- to 7-fold over whole chromatin. This was accomplished by selectively shearing chromatin with DNase II followed by fractionating the released portion on the basis of its solubility properties in 2 mM $MgCl_2$. The resulting soluble material comprises 11% of the total chromatin DNA and is impoverished in histone and enriched in nonhistone protein. Compared with unsheared chromatin, this minor fraction exhibits marked differences in chromosomal protein species. DNA renaturation studies indicate that this fraction is composed of a specific subset of whole genomal DNA sequences. Furthermore, DNA·RNA hybridization experiments suggest that almost 60% of the nonrepetitious DNA sequences of this minor fraction could code for cellular RNA.**

Differentiated eukaryotic cells transcribe a limited and tissue-specific portion of their nuclear DNA sequences (1, 2). It is now well established that transcription is restricted in isolated chromatin (3). Furthermore, recent evidence suggests that at least some of the mechanisms of genetic regulation remain intact in isolated chromatin (4, 5). At present, however, no conclusive data are available to indicate which components of chromatin serve as specific regulators of genetic activity. A direct approach to this question would be to study the components of the minor portion of chromatin that is transcriptionally active. This, however, requires as a prerequisite the development of chromatin fractionation techniques.

Two principal problems are encountered in designing a strategy for the fractionation of chromatin. First, to avoid cross-contamination, chromatin must be sheared to a size less than that of the average unit of transcription. Ideally, this should be accomplished by a method that does not lead to protein denaturation, rearrangement, or dissociation. Second, a gentle method of physically separating "active" from "inactive" material is required. Several groups have introduced fractionation techniques based on mechanical shearing followed by separation by differential centrifugation or column chromatography (6–10). An alternative method suggested by workers in our laboratory utilizes DNase II for shearing, and selective precipitation by mono- or divalent cations for chromatin fractionation (11, 12).

Previous studies have shown that DNase II preferentially attacks a minor portion of chromatin DNA; the amount of this "DNase-labile" fraction varies depending on the chromatin source but corresponds directly to the ability of the given chromatin to serve as a template for exogenous RNA polymerase (13). Furthermore, fractionation experiments using chromatin prepared from hepatoma cells pulse-labeled with [^{3}H]uridine reveal that over 60% of the label fractionates with 10% of the chromatin DNA (13). These observations prompted us to examine this technique of chromatin fractionation in more detail. In the present communication we report that rat-liver chromatin has been fractionated into a predominantly "active" component that differs in at least five ways from whole chromatin: chemical composition, chromosomal protein populations, template activity for support of RNA synthesis, DNA sequence complexity, and DNA sequence homology with cellular RNA.

METHODS

Chromatin Fractionation. Rat-liver chromatin purified by sucrose gradient centrifugation (3) was washed once with 10 mM Tris·HCl (pH 8) and dialyzed overnight at 4° against 200 volumes of 25 mM sodium acetate buffer (pH 6.6). The volume of the dialysate was adjusted to give an $A_{260\,nm}^{1\,cm}$ of 10 (measured in 0.9 N NaOH). The solution was brought to 24°, and DNase II (Worthington, HDAC) was added to 100 units/ml. The reaction was terminated after 5-min incubation by the addition of 50 mM Tris·HCl (pH 11) to pH 7.5 and cooling on ice. Unsheared chromatin (P1) was removed by centrifugation at 27,500 × g for 20 min at 4°. To the supernatant one ninety-ninth volume of 0.2 M $MgCl_2$ was added dropwise with stirring at 4°. After 30 min of additional stirring, the suspension was centrifuged as above yielding a pellet (P2) and supernatant (S2) fraction.

Chromatin Protein Analyses. Histone and nonhistone protein were determined as described (3). Acid-extracted protein was purified by ethanol precipitation and analyzed by disc electrophoresis as reported elsewhere (14). The acid-insoluble chromatin residue was homogenized in and dialyzed against 2.5% (w/v) sodium dodecyl sulfate–65 mM Tris·HCl (pH 6.8)–2% 2-mercaptoethanol. Samples were heated for 1 min before electrophoresis (15). Gels were scanned at 600 nm with a Gilford 2000 spectrophotometer.

DNA Reassociation Kinetics. DNA was purified from various chromatin fractions as described elsewhere (16). DNA isolated from the S2 chromatin fraction had a single-stranded length of 500 nucleotides, as determined by sedimentation velocity centrifugation under alkaline conditions (17). DNA isolated from total chromatin and from the P1 chromatin fraction was sheared by two passes through a Ribi-Sorvall

Abbreviation: C_0t, molar concentration of DNA nucleotides multiplied by time of incubation.

TABLE 1. *Properties of chromatin fractions*

Chromatin sample	% Chromatin DNA*	Template activity†	Composition relative to DNA (w/w): Histone protein‡	Composition relative to DNA (w/w): Nonhistone protein
Unfractionated	100	20	1.06	0.65
Fraction				
P1	84.6 ± 4.8	9	1.15	0.58
S2	11.3 ± 3.9	65	0.61	1.60
P2	4.1 ± 2.5	—§	—	—

* Mean of 11 determinations ± SD as estimated by absorbance at 260 nm.

† Percent template activity (24) as compared to DNA isolated from the same chromatin sample. *Escherichia coli* RNA polymerase (fraction IV) was prepared according to McConnell and Bonner (25).

‡ Histone protein determined from areas of densitometer scans of polyacrylamide gels (14) loaded with known amounts of proteins.

§ Not determined.

pressure cell at 50,000 lbs/inch^2. This procedure yields double-stranded fragments 400 to 450 nucleotides in length, as measured by electron microscopy (18). Kinetics of DNA reassociation were monitored by hydroxyapatite chromatography using standard techniques (19). Data obtained at various DNA and sodium ion concentrations were normalized to C_0t values equivalent to those obtained in 0.12 M phosphate buffer (0.18 M Na^+, see ref. 19). Computer analysis was performed according to Britten *et al.* (19).

Isolation and Labeling of Nonrepetitive DNA. DNA was incubated to an equivalent C_0t of 2.5×10^2 (S2 DNA) or 1.5×10^3 (P1 DNA), and the single-stranded fraction was isolated by hydroxyapatite chromatography. This material was dialyzed against distilled water, concentrated by lyophilization, dissolved in phosphate buffer, and allowed to renature as before. Finally, the DNA that remained single-stranded after two cycles of purification was incubated to a C_0t of 10^4. The resulting duplex material was dialyzed against distilled water and then concentrated. The purified nonrepetitive DNA was labeled with ^{125}I by the Commerford method (20) as modified in our laboratory (21). Specific activities of 1×10^6 cpm/μg were obtained.

DNA·RNA Hybridization. Total cell RNA from rat liver was isolated by a modified hot phenol–sodium dodecyl sulfate extraction procedure (22) and sheared by two passes through the Ribi-Sorvall pressure cell at 30,000 lbs./inch^2. The resulting RNA had an average length of 1000 nucleotides, as judged by sedimentation velocity centrifugation under nondenaturing conditions (23). Hybridization experiments were performed at 74° in 30 mM sodium phosphate buffer (pH 6.5)–0.675 M NaCl–1 mM EDTA at an RNA:^{125}I-labeled DNA mass ratio of 2 to 2.5×10^4:1 and an RNA concentration of 20 mg/ml. Samples were incubated in sealed capillary tubes. Reactions were terminated by a 20-fold dilution in reaction buffer at 60° followed by application to hydroxyapatite columns equilibrated with 30 mM phosphate buffer–0.1 M NaCl at 60°. Under these conditions both single- and double-stranded nucleic acids are absorbed while free ^{125}I passes through. Single-stranded DNA and the majority of the RNA were eluted with 0.12 M phosphate buffer; DNA·RNA duplexes were eluted with 0.48 M phosphate buffer. About 20% of the sheared RNA also eluted in 0.48 M phosphate buffer. The single-stranded and hybrid fractions were precipitated with 10% trichloroacetic acid after addition of 40 μg of bovine-serum albumin per sample. The amount of DNA in DNA·RNA hybrids was determined by collecting the resulting precipitates on membrane filters, and counting dried filters in a toluene-based scintillant. At zero time of incubation, about 2% of the ^{125}I-labeled DNA eluted from hydroxyapatite in the 0.48 M phosphate buffer fraction; this background value was subtracted from all time points. DNA·DNA reassociation, estimated by incubation of ^{125}I-labeled DNA with NaOH-hydrolyzed RNA, was not detectable above zero-time binding.

RESULTS

Fractionation of Chromatin Components. The experiments described herein have utilized a standard set of conditions for chromatin fractionation (see *Methods* for details). Chromatin is first selectively sheared by incubation with DNase II for 5 min. Unsheared chromatin is removed by centrifugation, yielding a pellet termed P1. This fraction comprises 85% of the input DNA (Table 1). The resulting supernatant (S1) is fractionated on the basis of its solubility in 2 mM $MgCl_2$ into a second supernatant fraction (S2) and a minor insoluble fraction (P2). The second supernatant fraction (S2) comprises 11% of the input DNA. Fraction P1 has a chemical composition not unlike that of unfractionated chromatin (Table 1). In contrast, fraction S2 shows a great enrichment in nonhistone protein and a depletion in histones. Fraction P2 comprises only a trace of material under the present conditions of fractionation; earlier studies using conditions resulting in more extensive shearing have shown that fraction P2 consists of DNA complexed stoichiometrically with histone (13). Template activity assays with exogenous polymerase reveal that fraction S2 chromatin is 3-fold superior to unfractionated chromatin as template for RNA synthesis (Table 1). In contrast, the template activity of fraction P1 is less than that of whole chromatin.

Fig. 1 shows the disc electrophoretic profiles of the chromosomal proteins of fractions P1 and S2. The populations of histone and nonhistone proteins of fraction P1 are similar to those of unfractionated chromatin (data not shown), as one might predict, since the majority of chromosomal protein (about 85%) remains in this fraction. In contrast, the proteins of fraction S2 are quite different. Histone I is absent and histone IV is present in a reduced proportion (Fig. 1*A*). A protein band migrating slightly slower than histone I appears to be enriched in fraction S2. The nature of this component is unknown, although a band at a similar position in whole rat-liver histone preparations has been shown to have a turnover rate at least 10-fold greater than histone I protein (26). Nonhistone polypeptides of fraction S2 show striking qualitative and quantitative differences as compared to those of fraction P1 (Fig. 1*B*). It is of interest that fraction S2 is rich in two polypeptides in the molecular weight range of 38,000, the approximate size of the subunits involved in the packaging of heterogeneous nuclear RNA (27).

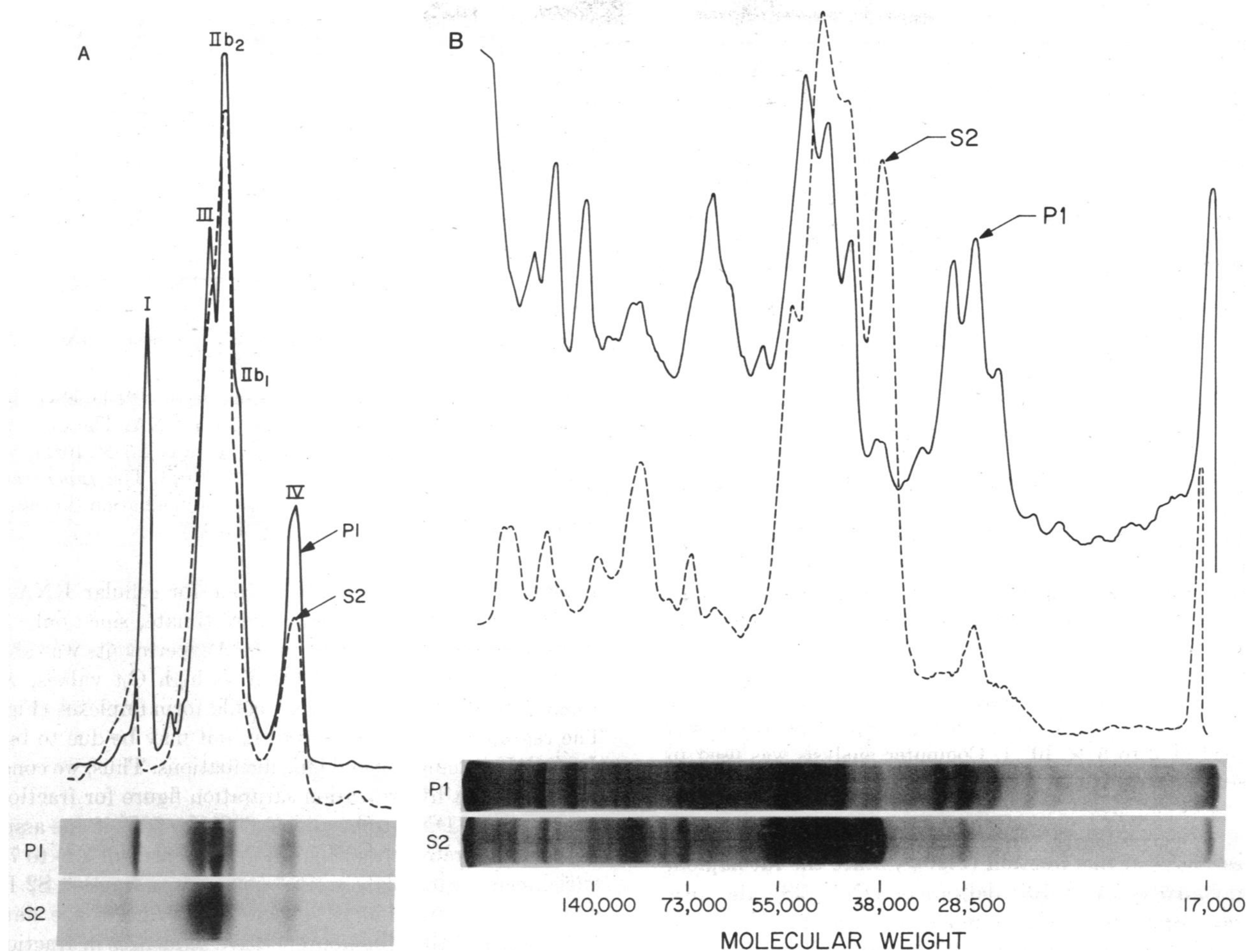

FIG. 1. Disc electrophoretic profiles of the proteins of chromatin fractions P1 and S2. (*A*) Histone protein. Acid-soluble protein (50 μg and 25 μg) from chromatin fractions S2 and P1, respectively, were separated by urea–disc gel electrophoresis. (*B*) Nonhistone chromosomal proteins. Nonhistone protein (100 μg) of each fraction was separated by sodium dodecyl sulfate–disc gel electrophoresis.

DNA Renaturation Kinetics. The kinetics of reassociation of unfractionated chromatin DNA and of fraction S2 DNA are presented in Fig. 2. Both samples have rapidly, intermediately, and slowly renaturing kinetic components, representing approximately 10%, 20%, and 70% of the input DNA, respectively. For unfractionated DNA these components correspond to highly repetitive, moderately repetitive, and nonrepetitive sequences, and agree both in amount and kinetic complexity with published data (21). In contrast, the rate of renaturation of fraction S2 DNA is strikingly different in that both the intermediately and slowly reannealing kinetic components have significantly lower $C_0t_{1/2}$ values than those of whole genomal DNA. If fraction S2 DNA were derived from a random population of chromatin DNA sequences, its reassociation curve would be identical to that of unfractionated DNA. The fact that both the intermediately and slowly reannealing components of fraction S2 DNA reassociate faster than those of unfractionated DNA clearly indicates that this material contains a specific subset of the sequences of the rat genome. The reassociation curve for fraction P1 DNA is nearly identical to that for unfractionated DNA (data not shown); this is reasonable since fraction P1 comprises 85% of the chromatin DNA.

It can be shown both by calculation and by experimentation that the slowly reannealing kinetic component of fraction S2 DNA corresponds to nonrepetitive DNA and not to some repetitive component. Fraction S2 contains 11.3% of the total chromatin DNA (Table 1), and its slow kinetic component

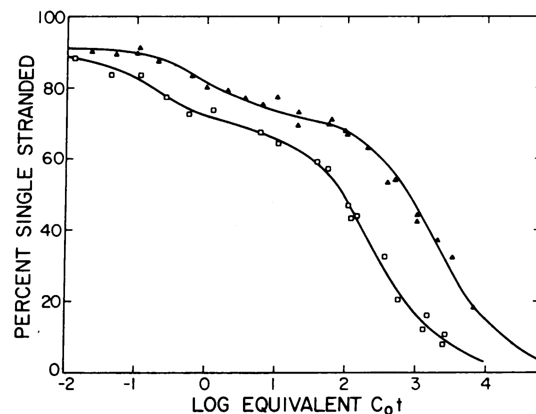

FIG. 2. Reassociation profiles of unfractionated chromatin DNA and fraction S2 DNA. Renaturation of total chromatin DNA (▲) and fraction S2 DNA (□) was assayed by chromatography on hydroxyapatite (19). The chromatin DNA points fall on the computer fit line (*solid line*) of the data of Holmes and Bonner (21). The line through the data for fraction S2 DNA was obtained by a similar computer analysis.

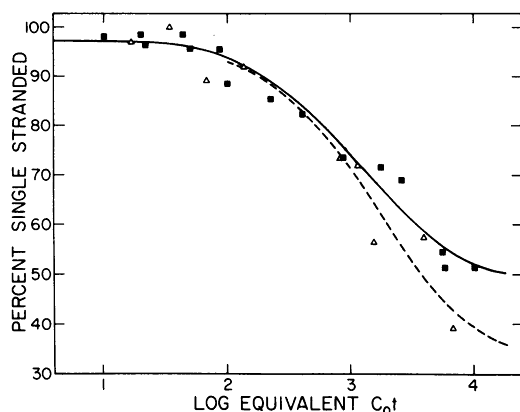

FIG. 3. Reassociation profiles of isolated nonrepetitive ^{125}I-labeled DNA in the presence of an excess of unfractionated DNA. Labeled nonrepetitive DNA (see *Methods*) of fraction S2 (■) or fraction P1 (Δ) was mixed with unlabeled total chromatin DNA in 30 mM phosphate buffer–0.675 M NaCl–1 mM EDTA, denatured for 5 min at 100°, and allowed to renature at 74°. The fraction of DNA in duplex was assayed by hydroxyapatite chromatography as described in *Methods* (section on RNA·DNA hybridization). The scale on the *abscissa*, equivalent C_0t, refers to the concentration of unlabeled DNA. Data were obtained at various concentrations of unlabeled DNA; however, the mass ratio of unlabeled DNA to labeled nonrepetitive DNA was maintained at 2 to 5 × 10^2:1. Computer analysis was used to fit lines to the data (19).

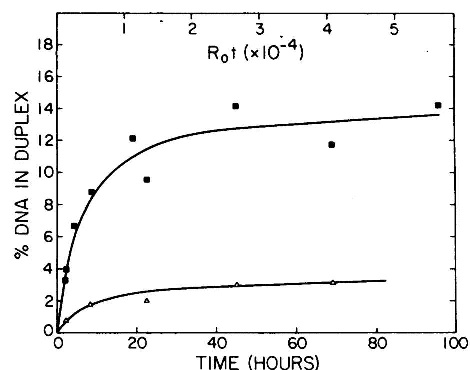

FIG. 4. Hybridization of nonrepetitive ^{125}I-labeled DNA from fractions P1 and S2 to sheared liver RNA. The mass ratio of RNA:DNA for fraction S2 DNA (■) was 2.5 × 10^4:1, while that for fraction P1 DNA (Δ) was 2.0 × 10^4:1. The *upper scale* on the *abscissa*, R_0t, is the product of time of incubation (in seconds) and molar concentration of RNA (59 mM).

comprises 63% of this fraction (Fig. 2). Since the rat haploid genome contains 1.8 × 10^{12} daltons of DNA (28), the analytical complexity of the slow component of fraction S2 DNA is (1.8 × 10^{12}) (0.113) (0.63) = 1.29 × 10^{11} daltons. The observed kinetic complexity of this component is 1.25 × 10^{11} daltons (Fig. 2; relative to *Escherichia coli*, $C_0t_{1/2}$ = 4.1). Thus, each sequence is represented approximately once in this kinetic portion of fraction S2 DNA. That this is indeed the case has been demonstrated directly by isolating this component (see *Methods*), labeling it *in vitro* with ^{125}I, and reannealing it in the presence of a vast excess of unfractionated chromatin DNA (Fig. 3). The $C_0t_{1/2}$ observed for such labeled S2 DNA was 1.3 × 10^3. Similarly, the $C_0t_{1/2}$ observed for the isolated nonrepetitive component of fraction P1 DNA was 1.5 × 10^3 (Fig. 3). The moderately repetitive sequences of fraction S2 DNA also represent a subset of the repetitive sequences of the genome. This matter will be discussed in detail elsewhere (Gottesfeld *et al.*, in preparation). At present it is not clear whether the fast reassociating component of fraction S2 DNA is analogous to highly repetitive DNA sequences, or whether it represents DNA fragments containing internal complementary sequences.

DNA·RNA Hybridization. The isolated nonrepetitive ^{125}I-labeled DNA of Fig. 3 was also hybridized to sheared, total liver RNA under conditions of vast RNA excess (Fig. 4). The saturation values, estimated from double-reciprocal plots of the data, were 3.5% and 14.5% for fraction P1 and S2 nonrepetitive DNA, respectively. In a similar experiment, 12.5% of fraction S2 nonrepetitive DNA hybridized to unsheared total liver RNA. The value of 3.5% obtained for fraction P1 is in accord with published values for the extent of transcription of nonrepetitive sequences in mouse-liver tissue (1, 2). These data suggest that fraction S2 DNA is enriched approximately 4-fold in sequences that code for cellular RNA. We consider this value to be an underestimate, since only 50% of the fraction S2 DNA used in these experiments was able to renature to whole genomal DNA at high C_0t values, while about 70% of fraction P1 DNA could form duplexes (Fig. 3). The reasons for this are unknown, but may be due to breakdown during handling and long incubations. Thus, we consider the true RNA hybridization saturation figure for fraction S2 nonrepetitive DNA to be almost 30% (or 60% if one assumes asymmetric transcription). This would represent a 6- to 7-fold enrichment in template active sequences in fraction S2 DNA over those in fraction P1 DNA. Furthermore, the data of Fig. 2 suggest that the nonrepetitive sequences of fraction S2 have been enriched by a similar factor [($C_0t_{1/2}$ total single copy DNA) ($C_0t_{1/2}$ S2 single copy DNA)$^{-1}$] = (1500) $(225)^{-1}$ = 6.7], in agreement with this prediction.

DISCUSSION

Based on several criteria, the technique we have adopted for chromatin fractionation appears to be successful. First of all, the sequences of DNA found in fraction S2 consist of a specific subset of the total genomal sequences. To the best of our knowledge, this is the first demonstration of such sequence fractionation by renaturation kinetics. Second, the nonrepetitive sequences of fraction S2 DNA are enriched in those which code for cellular RNA. This finding provides firm evidence for the partial purification of "template active" chromatin, originally suggested by experiments on the cofractionation of nascent RNA (13). Finally, chromosomal proteins have been fractionated both in a quantitative and qualitative sense.

Our success in the isolation of "template active" chromatin can be estimated in at least two ways: from saturation values obtained by DNA·RNA hybridization experiments and from assay of template activity *in vitro*. Both measurements yield values of 50–65% purity of "template active" chromatin in fraction S2. DNA reassociation studies on fraction S2 show a 6- to 7-fold enrichment of single-copy sequences over those of whole chromatin. Since 50–65% of these sequences are presumably active in RNA synthesis, isolation of pure template active chromatin would require a 9- to 14-fold purification over whole chromatin. This suggests that some 7–11% of whole genomal DNA is transcriptionally active. This estimate is in accord with published RNA hybridization data (2) for

the extent of genetic activity in liver if one assumes asymmetric transcription (3.5–5.5%). It should be noted that the contamination of fraction S2 DNA with transcriptionally inert sequences is not a random process, for if it were, the reassociation profile of fraction S2 DNA would not follow simple second-order kinetics as shown in Fig. 2.

Our findings suggest wide opportunities for the application of this technique in the study of the control of gene expression. One is the enrichment (by almost one order of magnitude) from whole chromatin of those nonrepetitive DNA sequences that are expressed in that chromatin. A second is the direct comparison of DNA sequence expression in the chromatin of different tissues, organs, or developmental states. A third might be in the study of the fidelity of reconstitution of chromatin from its several constituents.

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