Characterization of Pituitary and Peptide Hormones by Electrophoresis in Starch Gel*

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In 1957, Poulik (1) described a modification of Smithies' (2) method of starch gel electrophoresis incorporating a discontinuous buffer system. In his original presentation, Poulik described a peculiar and as yet not fully explained phenomenon of an advancing borate "front" which seemed to be a major factor responsible for the high degree of separation and resolution. In 1960, Ferguson and Wallace (3, 4), using a slightly different buffer system, substituting lithium hydroxide for the sodium hydroxide of Poulik's buffer system, and using thin 3-mm horizontal sheets of starch gel with high voltage under refrigeration, described a method of remarkably high resolution for the separation of pituitary peptides. Further evidence of the high degree of resolution of this method was the observation of Morris (5) that "pure" A1 hemoglobin, which produced a single benzidine-positive component by the more usual starch gel electrophoresis, was separated by the Ferguson and Wallace method into four distinct benzidine-positive components and two other benzidine-negative bands.

Our main interest in this method has been to follow the purification of pituitary hormones, and it has been most useful for this purpose. This report deals primarily with a description of the method and the characteristics of a variety of peptide hormone preparations thus far examined.

**EXPERIMENTAL PROCEDURE**

Partially hydrolyzed potato starch in a concentration of 14 g/100 ml (range 13 to 16 g/100 ml) was suspended in buffer in a vacuum flask immersed in a boiling water bath. The gel buffer was made up of 90 parts of 0.016 M Tris-0.0033 M citric acid, and 10 parts of 0.02 M lithium hydroxide-0.076 M boric acid. The pH was 8.0. Slight variations in pH of the gel buffer were made in some experiments without significantly altering the ionic strength to examine peptides isoelectric at pH 8.0 and to confirm or disprove apparent similarities of peptides in different preparations at pH 8.0. The initial starch suspension was agitated by hand until it became partially translucent. The gel was then either immediately covered with a heavy piece of plate glass (with a thin film of mineral oil on the surface) to insure a gel of uniform 3-mm thickness, or the gel was allowed to cool at room temperature for 3 to 5 minutes and then covered with Saran Wrap. In the latter preparation, the gel was 4 to 4.5 mm in thickness. The gel was cooled at 4° before use to facilitate handling when the samples were inserted.

Filter paper strips (Whatman No. 3MM), 16 x 3.0 to 4.5 mm, were moistened with the solutions under study and inserted into the starch along a line 8.0 cm from the cathode. Approximately 0.05 ml was absorbed by 1 cm² of filter paper. Solutions with a protein concentration range of 5 to 50 mg per ml were usually employed. The crude pituitary preparations containing multiple components were highly concentrated, whereas most of the highly purified preparations were satisfactorily developed with more dilute solutions. Then the gel was compressed toward the line of application by a ¼-inch glass shive placed behind the cathode end of the gel, to insure proper contact at the origin and to compensate for shrinkage as the borate "front" progressed. During electrophoresis the gel was covered either by a ¼-inch plate glass or by Saran Wrap with 1.5 cm of gel left uncovered at each end to receive the wicks. The electrode vessels contained a lithium borate buffer at pH 8.0 (0.1 M lithium hydroxide-0.38 M boric acid). Contact with the gel was made by folded cloth towelling soaked in the electrode buffer. When the current had been passed for 1½ to 2 hours the borate "front" had advanced to a position of 12 to 16 cm from the origin toward the anode. This selection of the site of origin and length of run gave optimal separation and resolution of the many acidic and basic components investigated.

During electrophoresis, the gel was cooled by a plate glass at −5°. A current of 100 to 120 ma was maintained by application of 800 to 1500 volts with a range of 24 to 44 volts per cm of starch. The voltage was increased in steps during electrophoresis to maintain this current as resistance increased with the advance of the borate ions.

A 0.5% solution of water-soluble nigrosin in methanol-acetic acid-water (30:10:40) was the most satisfactory stain for these studies; a variety of other protein stains and fixatives proved disappointing. Because the gels were poured in trays of plate glass, and care was taken to avoid any evaporation of buffer from the undersurface of the gel, it was not necessary to slice the gels for satisfactory staining, and usually only the undersurface of the gel was stained. However, slicing was easily accomplished with the 4.5-mm gels by a piano wire held taut in a hack saw; 1.5 mm of the upper layer were removed, and its cut surface was stained. This stained cut surface was some-
what superior to the stained undersurface of the gel as there was better resolution of closely spaced peptides. Evaporation caused crusting of the upper surface and unsatisfactory staining. The gels were immersed in the staining solution for 30 minutes and then left overnight in weaker stain to produce maximal contrast. They were washed several times with the solvent alone before photographing.

The various segments of the gel were eluted for biological studies by syneresis after freezing and identification of the bands was facilitated by staining a small contiguous area. Serrations helped identify the desired areas. Measurement of eluted protein was made by the Folin method of Lowry et al. (5) or by ultraviolet absorption.

RESULTS

Human Growth Hormone and Ovine Prolactin

Human growth hormone and ovine prolactin exhibited very similar patterns electrophoretically (Fig. 1), an observation also made by Ferguson and Wallace (6). Five different human growth hormone preparations, made by dissimilar methods but all considered highly purified, were examined, and each contained four major bands in common. The relative staining intensities of the corresponding bands were also similar and were virtually identical electrophoretically with the four major bands of three different sheep prolactin preparations. These major bands of growth hormone were not separated from those of prolactin even when mixtures of human growth hormone and prolactin were inserted at the cathode and electrophoresis continued until the borate "front" had traversed the entire length of the gel; this behavior was observed at both pH 8.0 and 8.5.

Both the human growth hormone and the prolactin preparations contained several other minor components with dissimilar electrophoretic mobilities. Besides these bands, there was a diffuse background staining in the area of the major peptides and trailing back to the origin, which was more intense with the preparations of growth hormone; roughly twice the protein concentration of growth hormone as prolactin was applied to produce the same staining intensity of the major bands. This electrophoretic similarity is of particular interest because Lyon (7) has reported that prolactin activity is contained in the human growth hormone preparations he has assayed to date, and conversely there has also been at least one report (8) that "pure" prolactin preparations have an anabolic effect in man.

Ferguson and Wallace reported that each of the four major peptides in N.I.H. prolactin contained prolactin activity (3), as shown by elution and crop sac assay. They also assayed the major peptides in the human growth hormone preparation of Raben for somatotrophic and prolactin activity and found each to contain both activities (6). Our own assays of human growth hormone after electrophoresis showed somatotrophic activity (growth rate of the hypophysectomized immature rat) only in the segments containing the major bands.

Immunological techniques thus far have not shown any interaction between these two preparations. Hayashida (9) found that antisera prepared to purified ovine prolactin did not cross-react with human growth hormone in vitro or in vivo, and Irie and Barrett (10) showed that antisera to human growth hormone did not cause agglutination of prolactin-coated red cells. Immunodiffusion on Ouchterlony plates with sheep prolactin and antisera to human growth hormone failed to produce a precipitin line.

Electrophoretic examination of a human prolactin preparation of Wilhelmi (Fig. 2) showed only two major bands. These had the identical mobility at pH 8.0 as the two slower moving major bands of ovine prolactin and human growth hormone, and many faintly staining minor bands could be seen, two of which corresponded to the two faster moving major peptides of sheep prolactin and human growth hormone. This particular human prolactin preparation was assayed to contain 0.17 unit per mg of growth hormone. Immunological studies in our laboratories showed an interaction between this preparation and human growth hormone.

Human Growth Hormone and Serum Proteins

Hayashida's immunological studies with Raben's preparation of human growth hormone suggested minimal contamination with a serum protein which he thought was probably albumin. It was observed that human serum albumin and the slowest moving major band in human growth hormone had similar electrophoretic mobilities at pH 8.0 (Fig. 3). With the gel buffer at pH 8.5, the relative mobility of albumin was increased and clearly separated from the human growth hormone peptide in question. It thus seems that none of the growth hormone components developed by starch gel electrophoresis represents albumin. On the contrary, with the use of antisera to human growth hormone, immunoelectrophoresis of normal human serum with or without added growth hormone yielded a fine but constant precipitin line in the area of the slower moving components of α₁, α₂, and β-globulins not seen after immunoelectrophoresis of human growth hormone alone. This suggests that the serum protein contaminant in the Raben preparation of human growth hormone is probably one of these serum proteins rather than albumin and is present in insufficient amount to be detected by starch gel electrophoresis or immunoelectrophoresis of human growth hormone alone.

Human Growth Hormone and Growth Hormone Preparations from Other Species

When human growth hormone was compared with simian and porcine growth hormone, all prepared by the Raben method (10), it was found that the latter two preparations shared three of the major components with the human preparation (Fig. 4), and similar methods (pH change, prolonged run, and mixing) as described for prolactin confirmed their electrophoretic similarity. The simian preparation showed an additional slower moving anionic major component that could not be seen in the human preparation, and this same band was present in the porcine preparation. The preparation of porcine growth hormone, besides showing the bands that it had in common with the simian and human preparations, exhibited three other slower moving anionic components not contained in the other two preparations. The finding of similar components in the human and simian preparations is consistent with their immunological and physiological properties. However, the observation that the porcine preparation displayed some bands in common with the simian and the human preparation is difficult to reconcile with the observation that it did not interact with the human or monkey preparation and with the earlier findings of its inactivity.
Figs. 1-12, 14-18
in man. It is of some significance perhaps that the background staining material in the simian and human preparations is similar in intensity and distribution, whereas in the porcine preparation it is less intense and distributed mainly near the origin. None of these preparations had any material, staining with nigrosin, on the cathode side of the origin, and segmental elution nonstaining protein migrating toward the cathode.

A1 and AZ and α-MSH are indicated by the markers in the α fraction. Staining material in the simian and human preparations is similar in intensity and distribution, whereas in the porcine preparation it is less intense and distributed mainly near the origin. None of these preparations had any material, staining with nigrosin, after starch gel electrophoresis and had maximal adsorption at 288 μm.

The only sheep growth hormone preparation examined contained a single major cationic peptide and multiple anionic components many of which had mobilities identical with the major peptides of the human, monekey, and pig preparations.

A purified preparation of bovine growth hormone of Wilhelm showed only a single major cationic band and shared no bands in common with the above preparations, although there was a considerable amount of anionic “backgroundlike” material near the origin and several minor anionic bands in the same area. The crude bovine growth hormone preparations studied were almost as heterogeneous as crude acetic acid extracts of pituitary glands containing upwards of 30 major and minor components moving both toward the anode and the cathode.

Melanocyte-stimulating Hormone and Corticotropin

The characteristic mobilities of α-MSH and β-MSH and a variety of corticotropin preparations are shown in Figs. 5 to 11. The major peptide in a preparation of α-MSH (Fig. 5) at pH 8.0 moved rapidly toward the cathode. A number of other minor cationic components were seen in this preparation prepared by Schally and Guillemin (11) but were poorly reproduced in the photograph. The synthetic α-MSH preparation of Hofmann, Yajima, and Schweitz (12) had an electrophoretic mobility identical with that of the naturally occurring tricapeptide. Also shown in the figure is a crude acetic acid extract of pig anterior pituitary glands that remained after Oxyceg adsorption of corticotropin and most of the MSH. It appeared that a considerable amount of α-MSH remained in this fraction.
or at least there was a major peptide present with similar electrophoretic mobility. It is from this crude extract that we have purified Peptides I and II (13), which are highly active in fat mobilization in the rabbit (14).

The β-MSH preparation of Schally (Fig. 6) contained at least one cationic and a number of major anionic components. The heaviest band, however, was not seen in the crude acetic acid extract indicating the efficiency of Oxycel in adsorbing this component.

Oxycel-purified corticotropin (15) (Fig. 7) had a number of components including α- and β-MSH and corticotropin A1 and A2, all of which could be identified by their characteristic mobilities. Corticotropin A4, the first fraction to emerge upon chromatography with IRC-50 of the Oxycel material (16), contained both α-MSH and β-MSH and corticotropin A2 in significant amounts but little or no corticotropin A1. “Pure” corticotropin A1 and A2 (Fig. 8) prepared by the same purification procedure were shown not to be completely homogeneous. The A1 fraction contained a trace amount of corticotropin A2 and the A2 fraction, in addition to a trace amount of corticotropin A1, consistently contained a major component that moved more rapidly toward the anode. This component after elution from the starch had potent fat-mobilizing activity in the rat epididymal fat pad. It has not yet been assayed for corticotropic activity. When corticotropin was first purified with oxycellulose, it was observed that alkali produced a change in its electrophoretic and chromatographic behavior (17). It has subsequently been shown that the chemical difference between corticotropin A1 and A2 is the absence of an amide group in the A2 peptide (18). Corticotropin was treated for 10 minutes before electrophoresis with 0.1 N sodium hydroxide (Fig. 9). Estimates from the relative staining intensities indicate that approximately 90% of the A1 peptide was converted to the A2 peptide by this brief, mild exposure.

Further countercurrent distribution fractionation and purification of Oxycel corticotropin was accomplished by Shepherd et al. in 1956 (18). A number of these preparations have been examined (Figs. 10 and 11). The electrophoretograms showed the β1 and β2 fractions to be almost as heterogeneous as the Oxycel fraction itself. The δ1 fraction concentrated a significant amount of corticotropin A1 and, in addition, contained multiple other components including small amounts of corticotropin A2 and β-MSH. The β- and γ-MSH fractions proved to be a relatively purified preparation of corticotropin A1 and A2 as was the γ1 + γ2 fraction. Corticotropin A4 was the predominant peptide in the β fraction and corticotropin A1 the predominant peptide in the γ1 + γ2 fraction.

Parathyroid Hormone

The electrophoretograms (Fig. 12) of parathyroid hormone in progressive stages of purity showed that the purest preparation (PTH-C) (19, 20) was not homogeneous and was not completely dissimilar from the partially purified material before countercurrent distribution; three major cationic components and a number of minor anionic bands were visible. The partially purified material had the same positively charged components and two major bands that moved toward the anode, besides several minor anionic components. It appeared that the effect of countercurrent distribution, in further purifying this hormone, effectively eliminated the major anions but left many minor anions, and failed to discriminate between the major cations.

Purification of the partially purified material by gel filtration through Sephadex G-50 did little to improve the electrophoretic appearance of the starting material. The material that was applied to the starch gel was that most retarded by Sephadex G-50, i.e. the descending limb of the second peak (Fig. 13). It is this material that was said to be as active in calcium mobilization in the parathyroectomized rat as preparations made by countercurrent distribution.4

Relaxin

Recently both Cohen (21, 22) and Frieden (23) independently have reported on highly purified relaxin preparations, nearly homogeneous by countercurrent distribution and other criteria. Starch gel electrophoresis of these preparations (Fig. 14) showed both to be quite heterogeneous and in many respects dissimilar from one to the other; only a few major bands were common to the two preparations. If the activity could be pinpointed to a particular zone, this technique would be of value in directing and guiding further purification.

Insulin and Glucagon

Purified pancreatic hormone, recrystallized insulin, and glucagon were each found to contain two major components (Fig. 15). The insulin preparation, assayed to contain less than 0.2% glucagon, had other minor components but none with the electrophoretic mobility of the major bands of glucagon. It is of interest that the amino acid sequence of insulin was determined with even cruder preparations than the one in the electrophoretogram (24). Probably, one of the major bands in each preparation represents a substance with a minor chemical change in the parent molecule.

Some Characteristics of the Method

Observation of a mixture of acidic dyes during the course of electrophoresis showed the individual components to be sharpened by the borate “front” as it passed, and the speed of migration of all anionic components was increased with relatively greater mobility given to the normally faster moving ones. Albumin and other proteins behaved in a similar manner when observed prestained with bromphenol blue or nigrosin. Albumin (human serum diluted 1:10) and Peptide I (13) (Fig. 16) were inserted in the gel at four different origins. Those inserted nearer the cathode and thus under the influence of the borate area for the longer time moved the farthest; migration was progressively less the farther away the origin was removed from the cathode. The first three inserts nearest the cathode were sharper than the fourth which was not affected by the borate “front” at all. In each instance the third was sharper than either two or one as it was the last to be traversed by the “front” before the electrophoresis was completed.

Basic dyes (thionine and brilliant green) did not behave in the same fashion but were observed, in traveling toward the cathode, to become more blurred and indistinct after the borate front had passed. Cytochrome c behaved similarly. Also, the basic peptide components of some of the pituitary preparations were not as clearly and sharply defined as the acidic ones. Brilliant green, a basic dye, moved toward the cathode at a slow

4 H. Rasmussen, personal communication.
rate, and was sharply resolved. Once it was traversed by the borate "front," its speed of migration was increased, and it became spread out over a wider area and lost its sharp resolution.

A similar pattern of behavior was observed with a number of synthetic cationic peptides of the ACTH series prepared by Hofmann (12); they showed an increased rate of migration and blurring after the borate "front" had affected them (Fig. 17). Inserts of the octapeptide III-62, (His-Phe-Arg-Gly-Tyr-Asp-Pro-Val-amide) nearest the cathode moved considerably further toward the cathode and were considerably less highly resolved than the same material with more anodally advanced origins that had been affected for a shorter period of time or not at all by the borate "front." Synthetic α-MSH, a tridecapeptide, and naturally occurring basic peptides from crude anterior pituitary extracts that have not as yet been further characterized behaved similarly.

Although this method is somewhat less than ideal for the examination of basic peptides, we have shown clearly nonhomogeneity in two of eight cationic synthetic peptides of the ACTH-MSH series thus far examined. These were found to be homogeneous by other methods including paper chromatography and countercurrent distribution. The percentage of contaminating substances in these two preparations estimated by nigrosin staining intensity was quite small and quite concentrated solutions had to be applied to bring them out. One synthetic posterior pituitary peptide, oxytocin (Syntocin, Sandoz Chemical Works, Inc.), similarly examined, showed a single slowly moving cationic band.

The behavior of the major peptide component of a beef growth hormone preparation of Wilhelmi and of corticotropin A₁ was most puzzling (Fig. 18). When these materials were inserted into the gel at an advanced (anodal) origin and were affected by the borate "front" for a short period of time or not at all, they behaved as anions. When the origin was in a more normal position near the cathode, their direction of migration was reversed after the borate "front," and thus they appeared to behave as cations.

We do not have a ready explanation for this unusual behavior. However, in measurement of pH changes in the gel buffer (Fig. 19), after segmental elution at the completion of migration, it was noted that associated with the borate "front" but not preceding it the pH of the gel buffer was 0.2 to 0.3 unit higher than the starting pH. The unaffected buffer preceding the borate "front" was unchanged from its initial pH. The gel buffer trailing toward the cathode behind the borate "front" gradually returned to the initial pH. The highest pH values recorded were right at the front and in the trailing 2-ep segments.

These changes may explain in part the behavior of a preparation of cytochrome c, the major components of which were retarded in their migration toward the cathode by the borate "front;" if it is assumed that at pH 8.0 it was incompletely ionized, then at a higher pH it would have been even less ionized and its mobility impaired. The increased rate of migration of brilliant green and of the cationic peptides could not be explained by the change in pH but may be attributable to the increased potential drop in the borate-affected gel.

**Difficulties with Elution and Bioassay**

We have not had a high degree of confidence in the results obtained from the biological studies of single closely related peptides after elution for three reasons. (a) There was a significant amount of trailing of individual components even though there was not enough protein to be visible by staining. This was shown in one experiment when Peptide 1, known to be highly active in fat mobilization in the rabbit, was eluted, concentrated, and subjected to electrophoresis a second time. Significant fat-mobilizing activity was found for several centimeters behind the zone of staining although the only stainable material and the majority of the activity were in the area expected. For this reason, we were hesitant to ascribe biological activity to peptides in various preparations that were associated with a faster moving peptide with the same activity. (b) Peptides traveled at a different rate throughout the thickness of the gel, presumably because of temperature gradients. Thus, they moved more slowly on the undersurface of the gel, which was in closest proximity to the refrigerated plate. The least cooling was in the middle of the gel and the peptides moved fastest in this region. The upper surface of the gel was of an intermediate temperature and migration was at an intermediate speed. This phenomenon made it difficult accurately to cut out and elute a single peptide, particularly if it was close to other peptides even though they appeared adequately separated on one particular surface. (c) Where excessive evaporation of buffer caused crusting of the upper surface, albumin and other peptides gave a long trail under the crust. This was of such magnitude as to be visible by staining the upper cut surface at the same time that the stained bottom surface yielded its usual pattern with high resolution.

**Staining and Protein Distribution**

Attempts to stain these protein hormone preparations with protein stains other than nigrosin were disappointing, the peptide-dye complex seeming to wash out of the gel. We also had little success with a variety of fixative methods, including formalin, picric acid, heat, mercurial precipitants, trichloroacetic acid, and a variety of alcohols. Prior fixation of some pituitary preparations in 30% formaldehyde, however, did have the advantage of improving the appearance and bringing out cationic peptides not seen with nigrosin-staining alone; however, the opposite was its effect on acidic peptides. Simple aqueous or methanolic solutions of nigrosin were entirely unsatisfactory. A weak acetic acid solution of nigrosin had the advantage of preserving the translucency of the gel but was inferior to the methanol-acetic acid-water solvent in that far fewer bands could be brought out clearly.

A possible clue to the advantage of water-soluble nigrosin over other protein stains was obtained by electrophoresis of these...
materials themselves in the starch gel. Water-soluble nigrosin was separated into upwards of 25 different components, all moving toward the anode and ranging in color over almost the entire rainbow. None of the other protein stains so examined including alcohol-soluble nigrosin, Amidoblack 10B, Ponceau R, Ponceau S, brilliant green, thionine, and a number of others had as many different components by this method. They ranged from one to a maximum of five.

Water-soluble nigrosin itself, however, was not completely satisfactory in all instances, and its shortcomings were not overcome by substitution of any of the above noted stains including ninhydrin. Morris pointed out that preheating or formalin fixation before staining with nigrosin was necessary to bring out the various components in his preparations of thyrotropin, and Pierce had little success with stains other than Amidoblack 10B. Ferguson and Wallace (6) have reported, and we can confirm, that 2 to 4 times as much human growth hormone as sheep prolactin had to be applied to the starch to bring out the same intensity of staining. This would suggest that a considerable quantity of protein in the human growth hormone preparation was unstained or poorly stained.

Other similar examples of discrepancy between amount of material applied and staining intensity were noted. Synthetic oxytocin at a concentration of 10 mg per ml produced only one faint band, whereas most of the synthetic peptides of the ACTH series stained intensely when considerably less concentrated. The $\alpha$-MSH preparation of Schally at 5 mg per ml had as its major component a peptide with intense staining characteristics. $\alpha$-MSH at twice this concentration was stained less intensely.

The different intensities of staining and the possibility that some components may not stain at all left some uncertainties as to the degree of contamination of seemingly pure preparations.

Figs. 20 and 21 represent the segmental distribution of protein measured by the Folin reaction on eluates of two different preparations after starch gel electrophoresis. All growth hormone preparations (pig, monkey, and human) had a secondary peak near the anode. In none of these preparations was any material visible in the corresponding area of the stained gel. Bioassay and immunoassay of the human growth hormone segments indicated that this anodal secondary peak contained no growth hormone activity. Rather growth hormone activity was only found in the major peak which corresponded to the area where the major components of this preparation were seen after staining with nigrosin. On the other hand, the distribution of protein after electrophoresis of ovine prolactin and Peptides I and II corresponded well with their distribution by staining.

**SUMMARY**

A variety of pituitary and other protein hormone preparations exhibited characteristic patterns in starch gel electrophoresis, with the Ferguson and Wallace modification of Poulik's discontinuous buffer system. Five human growth hormone preparations prepared by different methods appeared similar. The four major bands in human growth hormone were inseparable from the four major bands in three different ovine prolactin preparations. Human prolactin shared two major bands in common with both human growth hormone and ovine prolactin. Human, simian, and porcine growth hormone shared three major components indistinguishable from one another, even with change in pH and prolonged electrophoresis. The simian preparation had one and the porcine preparation three major components not detected in the human preparation. Purified bovine growth hormone had no bands in common with the other three preparations.

Among the melanocyte-stimulating hormones (MSH), synthetic $\alpha$-MSH (Hofmann) had the identical electrophoretic mobility as the major component in Schally's preparation. The characteristic pattern of corticotropin $A_1$ and $A_2$ and $\alpha$-MSH and $\beta$-MSH was established; a variety of corticotropin preparations were examined, and the content of each of these peptides was assessed.

Three purified parathyroid hormone preparations and two purified relaxin preparations were found not to be homogeneous.
but showed many cationic and anionic components. Highly purified recrystallized insulin and glucagon both contained two major anionic peptides clearly separated from each other.

The peculiarities of the method are discussed, particularly as regards the borate "front," which seemed to be one of the prime factors responsible for the high degree of separation and resolution.

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