first to GMF and then to insulin, but not if they are exposed to these substances in the reverse order.

Homogeneous populations of rat astroblasts were obtained (*8*) and seeded sparsely in secondary culture in plastic wells in a base medium (a 1:1 mixture of medium F12 and Dulbecco's modified Eagle medium) supplemented with 10 percent fetal calf serum. One day later the cells were starved of serum for 48 hours and exposed to the base medium containing various additives (Table 1). When the culture was exposed to GMF for 4 hours, washed, and then exposed to insulin for 4 days, the mitogenic effect was similar to that of N2 combined with GMF. The effect was not observed if the culture was first exposed to insulin for 4 hours and then to GMF. Medium N2, insulin, or GMF alone did not have this effect. The minimum length of exposure to GMF necessary to elicit the effect was 2 hours (Fig. 1A). After GMF was removed, the culture had to be stimulated by insulin within 1/2 hour or mitogenicity was reduced (Fig. 1B).

The sequential relation described above suggests that GMF functions as a competence factor; namely, that it renders cells potentially able to leave the quiescent G_0 state and enter the cell cycle. Insulin, a progression factor (*9*), propels the cycle thereafter. Competence factors have to be present for a short time only, whereas progression factors must be continuously present for cells to proliferate (*9*). Pledger *et al.* (*10*) showed platelet-derived growth factor (PDGF) to be a competence factor. This growth factor differs from GMF in being a basic protein. While the minimum time required for cells to become competent in the presence of PDGF is about the same as in GMF, the competent state induced by PDGF is stable for at least 13 hours (*10*). The difference in stability could be due to the factors themselves, the cell type (PDGF was tested on BALB/c mouse 3T3 fibroblasts), or both.

Since GMF and glial cells are both found in the brain, elucidation of the mechanism of GMF action should help to show how the nervous tissue regulates the proliferation and maturation of its own cells.

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Appearance of a New Nucleosomal Protein During Differentiation of Human Leukemia (HL-60) Cells

Abstract. *A 60-kilodalton protein was identified in chromatin digested by micrococcal nuclease during retinoic acid–induced differentiation of human leukemia (HL-60) cells to mature-like granulocytes. The protein was not detected in a retinoic acid–resistant variant of the HL-60 cell line treated with retinoic acid, in HL-60 cells induced with dimethyl sulfoxide, or in normal human granulocytes. This protein may have an important role in the regulation of retinoic acid–induced leukemic cell differentiation.*

Human leukemias are viewed as clonal diseases resulting from blockage at specific stages of cell differentiation. A conceptual approach to treatment is to induce leukemic cells to undergo terminal differentiation and to inhibit their proliferation (*1*). Human promyelocytic leukemia (HL-60) cells (*2*) can be induced to differentiate to functionally mature granulocytes by a physiologically tolerable dose of retinoic acid (RA) (*3*). Induction of HL-60 cells to undergo differentiation may be triggered by RA-induced intracellular factors responsible for rearrangement in the macromolecular structure of chromatin. In this study the protein components of monomer nucleosomes (mononucleosomes) isolated from micrococcal nuclease–digested chromatin during RA induction were analyzed. A nucleosomal protein with a molecular weight of approximately 60 kilodaltons (kD) (protein P60) was identified that quantitatively corresponded to the extent of RA-induced HL-60 cell differentiation. Protein P60 was not detected in an RA-resistant HL-60 cell line (*4*), in HL-60 cells induced with 1.3 percent dimethyl sulfoxide (DMSO), or in freshly prepared human granulocytes.

Eukaryotic chromatin consists of a string of repeating subunits. Each subunit, termed a nucleosome, has DNA strands wrapped on the surface of a histone octamer core particle that is linked to other particles by internucleosomal DNA, called linker DNA (*5, 6*). The primary site of micrococcal nuclease degradation under limited conditions is on the linker DNA structures (*7*). Digestion of chromatin structure with micrococcal nuclease therefore results in the release of mononucleosomal particles as well as dimer, trimer, and oligomer particles from the chromatin string. These particles can be separated by sucrose density gradient centrifugation (*8*) or by polyacrylamide gel electrophoresis (*9*). We attempted to determine whether any change in mononucleosomal protein components occurs in HL-60 cells during RA-induced cell differentiation.

Approximately 40 and 95 percent of HL-60 cells (2×10^5 cells per milliliter) were induced to undergo terminal differentiation when cultured with RA (0.5 μM) or DMSO (1.3 percent) for 3 days and 6 days, respectively. The differentiated cells demonstrated morphological and functional maturation, as assessed by their ability to reduce nitroblue tetrazolium dye (*10*) and to phagocytose latex beads (*11*).

At various times cells (1×10^9) were harvested and fractionated. Nuclei were isolated and treated with 0.5 percent Nonidet P-40 before chromatin purification in a buffer system of low ionic strength (*12*). Approximately 15 percent of chromatin DNA was digested by micrococcal nuclease (the method is described in the legend to Fig. 1). The released nucleosomal particles were isolated by centrifugation in a linear density gradient of 5 to 28.8 percent sucrose. Three major categories of nucleosomes were separated in addition to the nondigested residual chromatin fragments that sedimented on the bottom of the gradient (Fig. 1). Superimposed sedimentation profiles obtained from noninduced cells and RA-induced cells revealed that monomer particles from cells induced for 6 days sedimented slightly faster than those from noninduced cells.

The monomer fractions were pooled

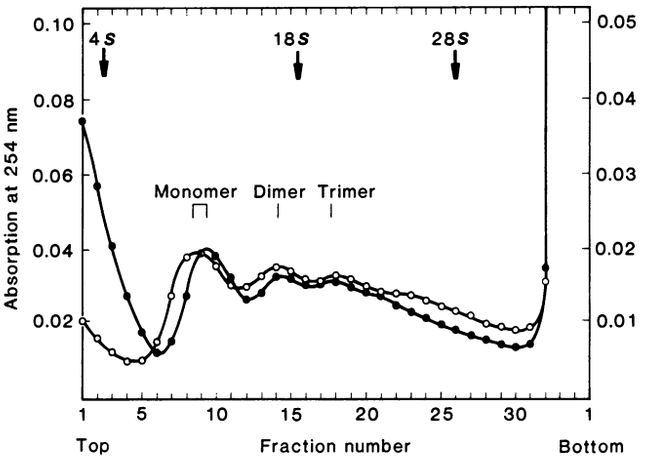

Fig. 1. Sucrose density gradient centrifugation of micrococcal nuclease–released nucleosomal particles. Chromatin was prepared from a total of 2×10^9 HL-60 cells (40 to 60 passages) in culture with or without the addition of RA (5×10^{-7} *M*). The cells were washed in buffer A (10 m*M* tris, *p*H 8.0 at 25°C; 1 m*M* $MgCl_2$; and 5 m*M* butyrate) plus 1 m*M* phenylmethylsulfonyl fluoride (PMSF) before being lysed with 0.5 percent Nonidet P-40 (*12*) and centrifuged at 10,000*g* for 10 minutes. Pellets were washed twice in buffer B (1 m*M* Na-Hepes, *p*H 7.5 at 25°C, and 0.1 m*M* PMSF) in the presence of 0.2 m*M* $CaCl_2$, and DNA (600 μg/ml) was incubated with micrococcal nuclease (30 unit/ml; Millipore) at 37°C for 5 minutes to digest about 15 percent of the total DNA. The enzyme reaction was stopped by chilling the solution in ice and adding 1 m*M* EDTA and 0.5 m*M* EGTA. Samples were centrifuged at 12,000*g* for 5 minutes. About 0.6 unit of each supernatant was layered over a linear density gradient of 5 to 28.8 percent sucrose (33 ml) containing 10 m*M* tris (*p*H 7.5 at 25°C), 1 m*M* EDTA, and 0.1 m*M* PMSF. Centrifugation was carried out at 26,000 rev/min and 4°C for 16 hours with a Beckman SW27 rotor. Gradients were fractionated by upward displacement with 40 percent sucrose and monitored at 254 nm. Monomers, dimers, and trimers were separated by this procedure. Svedberg (*S*) values for a set of tritiated transfer RNA markers run in parallel are indicated by arrows. The sedimentation profiles for noninduced cells (○) and for cells induced by RA for 6 days (●) were superimposed for comparison. One of three experiments is represented.

and adjusted with 2*M* NaCl to extract the nucleosomal protein components, which were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). Major protein bands included histones and a protein of about 54 kD (protein P54). An additional protein of 60 kD (protein P60) was revealed in cells induced with RA for 3 days and was more prominent in cells induced for 6 days. This protein was not detected in noninduced cells. The relative intensities of the protein bands from each sample were analyzed by scanning with a densitometer. Histone H3 was taken as the control protein band in each gel slot because it is believed to be one of the most stable proteins in nucleosomal structures (*13*). The intensity ratio of the protein P60 band to the histone H3 band (1.05) for cells induced for 6 days was about three times greater than the ratio (0.36) for cells induced for 3 days. Intensity in the same region of protein P60 in noninduced cells was virtually indistinguishable from the background. Moreover, the intensity of histone H2a had decreased 85 percent 6 days after the induction of differentiation.

Further analysis of the nucleosomal components was carried out by two-dimensional gel electrophoresis. Nuclease-released nucleosome particles were fractionated on a 5 percent polyacrylamide gel with a buffer system of low ionic strength (*12, 14*) as the first dimension. The procedure allowed separation of the two subgroups of mononucleosomes (MN1, mononucleosomal core particles containing DNA fragments of about 140 base pairs, and MN2, mononucleosomes containing DNA of 160 to 200 base pairs) and of a group of dimer nucleosomes (DN) containing DNA fragments of 270 to 400 base pairs. Identification of DNA fragment size was achieved by treating the first-dimensional gel with SDS to dissociate DNA-binding proteins followed by electrophoresis of the DNA fragments on a 9 percent polyacrylamide gel as the second dimension (*12*). Identification of nucleosomal protein components was carried out by the protamine displacement method (*15*). The protein components separated in the first-dimensional gel were dissociated by incubating the gel with protamine sulfate. The gel was then layered horizontally on a 15 percent polyacrylamide gel and electrophoresis was carried out with an acid-urea buffer system.

The two-dimensional pattern of proteins was revealed by a silver staining procedure (Fig. 3) (*16*). Patterns mapped were virtually identical between noninduced cells and cells induced for 6 days with RA except that a protein, designated X, which migrated more slowly than

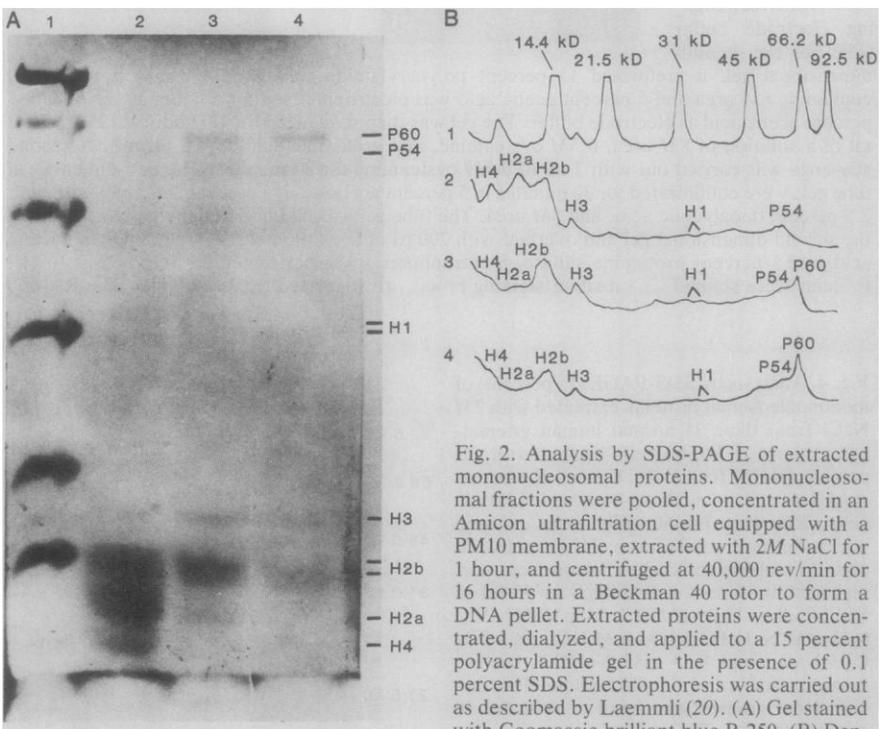

Fig. 2. Analysis by SDS-PAGE of extracted mononucleosomal proteins. Mononucleosomal fractions were pooled, concentrated in an Amicon ultrafiltration cell equipped with a PM10 membrane, extracted with 2*M* NaCl for 1 hour, and centrifuged at 40,000 rev/min for 16 hours in a Beckman 40 rotor to form a DNA pellet. Extracted proteins were concentrated, dialyzed, and applied to a 15 percent polyacrylamide gel in the presence of 0.1 percent SDS. Electrophoresis was carried out as described by Laemmli (*20*). (A) Gel stained with Coomassie brilliant blue R-250. (B) Densitometer scanning profile of the gel (1, molecular weight markers of lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B; 2, proteins from noninduced cells; 3, proteins from cells induced with RA for 3 days; and 4, proteins from cells induced with RA for 6 days). Relative intensities of protein bands were determined by measuring the area covered by each peak of densitometer scanning profiles; protein bands were designated as described in text.

histone H1, appeared in MN2 and DN particles of RA-induced, but not noninduced, cells. Another protein, designated Y, migrated slightly faster than protein X and appeared in MN2 and DN particles of both noninduced and RA-induced cells. This mapping procedure allowed us to identify all the histone subgroups as well as semihistone protein A24 and high-mobility group (HMG) proteins associated with nucleosomal structures (*12, 17*); it did not provide molecular weight information to enable us to determine whether proteins X and Y were proteins P60 and P54. Nevertheless, protein X was the only additional protein appearing in the nucleosomal protein map of cells induced to differentiate with RA.

The change in histone H2a revealed in one-dimensional SDS-PAGE was not observed in two-dimensional maps, perhaps because the displacement of proteins by protamine from nucleosomal particles is much more complete than that provided by extraction of proteins from particles with 2*M* NaCl. Therefore the difference in intensities of histone H2a bands from RA-induced and noninduced cells may not be distinguishable in gels stained with the sensitive silver procedure.

To determine whether the appearance of protein P60 in RA-induced cells is specifically related to RA treatment, we examined an RA-resistant HL-60 cell line (*4*). SDS-PAGE did not reveal protein P60 in 2*M* NaCl extractions of mononucleosomal proteins from resistant cells treated with RA for 6 days. In addition, protein P60 was not detected in HL-60 cells induced with 1.3 percent DMSO for 6 days or in freshly prepared normal human granulocytes (Fig. 4).

Since the amount of protein P60 appearing after RA induction corresponds to the extent of cell differentiation and since this protein was not detected in noninduced and RA-resistant cells, the expression of protein P60 may be an integral step in HL-60 cell differentiation. In addition, lack of protein P60 in normal human granulocytes and DMSO-induced HL-60 cells suggests that the appearance of protein P60 is unique to differentiation of RA-induced HL-60 cells. We do not exclude the possibility of very low levels of protein P60 being present in those cells in which protein P60 was not detected by our method. Therefore, although our interpretation of these data suggests that protein P60 is a newly expressed gene product activated by RA induction, we cannot rule out the possibility of protein P60 gene amplification occurring as a result of RA induction. It is not known whether the appearance of protein P60 is related to the diminishing amounts of histone H2a. Nevertheless, a unique nucleosomal H2a-specific protease that may be able to destabilize the nucleosome in vivo has been demonstrated (*18*). In addition, a recent report (*19*) indicates that degradation of histone H2a does occur during the differentiation of HL-60 cells, and this may be responsible for the decrease in histone H2a. The association of protein P60 with nucleosomal structures strongly suggests its importance in the rearrangement of chromatin structure during RA-induced cell differentiation. Since protein P60 appears during HL-60 cell differentiation, it may have an important role in regulating gene expression during that process.

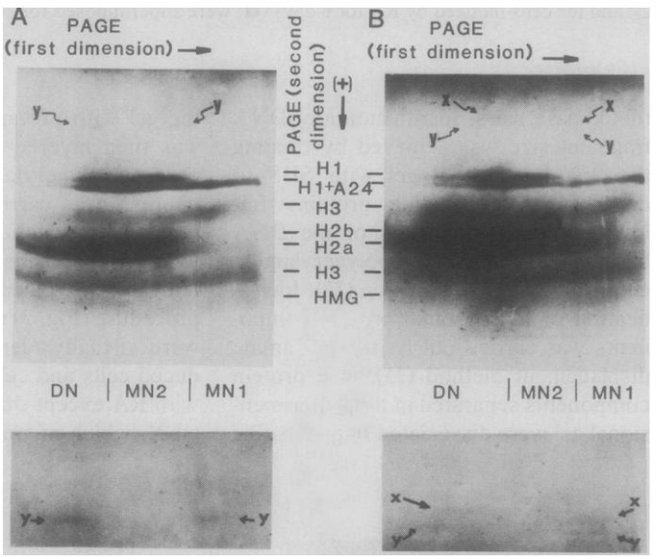

Fig. 3. Two-dimensional electrophoresis of nucleosomal proteins isolated from (A) noninduced HL-60 cells and (B) cells induced for 6 days with RA. Samples of micrococcal nuclease–digested chromatin were electrophoresed on 5 percent polyacrylamide tube gels buffered with a solution of low ionic strength (6.4 m*M* tris, *p*H 8.0 at 25°C; 3.2 m*M* sodium acetate; and 0.32 m*M* EDTA) for 15 minutes at 50 V followed by 4 hours at 75 V with recirculating electrode buffer (*15*). For the second-dimensional gel, a preformed 15 percent polyacrylamide slab gel (14 by 14 by 0.12 cm) containing 6*M* urea and 5 percent acetic acid was electrophoresed for 5 hours at 125 V with 5 percent acetic acid as electrode buffer. The gel was then scavenged for 1.5 hours at 125 V with 2 ml of a solution of 8*M* urea, 0.3*M* cysteamine, and protamine sulfate (0.3 mg/ml). A second scavenge was carried out with 2 ml of 0.6*M* cysteamine for 30 minutes. The first-dimensional tube gels were equilibrated for 30 minutes in 5 percent acetic acid, 5 percent 2-mercaptoethanol, 2.5 percent thioglycolic acid, and 8*M* urea. The tube gel was laid horizontally across the top of the second-dimensional gel and overlaid with 200 μl of a solution of 6*M* urea, 5 percent acetic acid, and 1 percent protamine sulfate. Electrophoresis was performed for 16 hours at 100 V. Proteins were stained with a silver staining procedure suggested by the supplier (Bio-Rad).

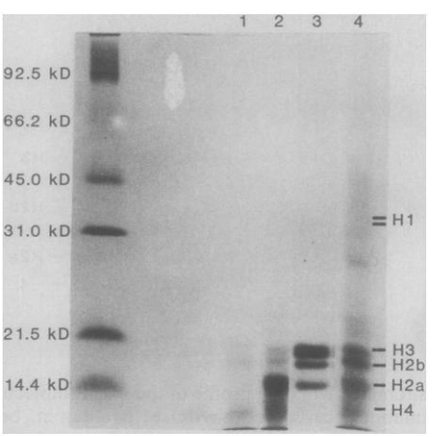

Fig. 4. Analysis by SDS-PAGE (15 percent) of mononucleosomal proteins extracted with 2*M* NaCl from (lane 1) normal human granulocytes, (lane 2) HL-60 cells treated with 1.3 percent DMSO for 6 days, (lane 3) RA-resistant HL-60 cells, and (lane 4) untreated HL-60 cells. Resistant HL-60 cells were cultured under the same conditions as HL-60 cells. To obtain human granulocytes, blood from normal healthy donors was diluted in 95 ml of SBSS buffer (137 m*M* NaCl, 74 m*M* KH_2PO_4, 0.42 m*M* NaH_2PO_4, 1.34 m*M* KCl, 18.3 m*M* $C_2H_3NaO_2$, and 8.33 m*M* $NaHCO_3$) before being applied onto a Ficoll-Hypaque (Pharmacia) linear gradient and spun at 400*g* for 30 minutes at 20°C. The pellet was saved, resuspended in 80 ml of SBSS and 1 percent dextran S-200 (Sigma), and centrifuged at 2*g* for 3 minutes. The supernatant was saved and cells were harvested and washed with SBSS twice. More that 90 percent of the cells harvested were granulocytes, as judged from smears on microscope slides stained with Wright-Giesma stain. SDS-PAGE was performed as described in the legend to Fig. 2.

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X-ray Laue Diffraction from Protein Crystals

Abstract. *In conventional x-ray diffraction experiments on single crystals, essentially monochromatic x-rays are used. If polychromatic x-rays derived from a synchrotron radiation spectrum are used, they generate a Laue diffraction pattern. Laue patterns from single crystals of macromolecules can be obtained in less than 1 second, and significant radiation damage does not occur over the course of an exposure. Integrated intensities are obtained without rotation of the crystal, and individual structure factors may be extracted for most reflections. The Laue technique thus offers advantages for the recording of diffraction patterns from short-lived structural intermediates; that is, for time-resolved crystallography.*

Structural intermediates in enzyme-catalyzed reactions or in the ligand binding reactions of myoglobin and hemoglobin typically have lifetimes at physiological temperatures of milliseconds or less. These transient intermediates cannot be crystallized and studied directly by x-ray crystallographic methods. Rather, their structures have been inferred from presumably similar, stable structures which can be crystallized, such as enzyme-product and enzyme-inhibitor complexes, complexes of hemoglobin with nonphysiological ligands, or hemoglobin locked in a single quaternary structure. In favorable cases, transient intermediates can be generated by photoactivation of a stable structure in the crystal, as in the photodissociation of carboxymyoglobin and carboxyhemoglobin by a brief light pulse and subsequent recombination with carbon monoxide in the dark (*1*). The crystal structures of photogenerated intermediates can be studied directly, provided that their lifetimes are long in relation to the minimum x-ray exposure time required to record a useful diffraction pattern. Even with an intense synchrotron x-ray source coupled to a conventional monochromator, a minimum exposure time of the order of minutes for an oscillation photograph is required (*2*). Although the lifetime of intermediates may be prolonged by cooling the crystal, the use of a monochromatic source requires that the crystal be rotated during the exposure in order to obtain integrated intensities from which quantitative Fourier structure amplitudes can be extracted. For exposures of less than about 1 second, unrealistically large angular velocities of the crystal would be required.

We now describe our experiments with an intense polychromatic x-ray source to generate a Laue diffraction pattern (*3*) and outline the underlying theory of these experiments. Instrumental and certain experimental aspects have been described (*4*). Although we focus on its applications to macromolecular crystallography, the Laue technique is equally applicable to chemical crystallography (*5*), solid-state physics, and surface diffraction.

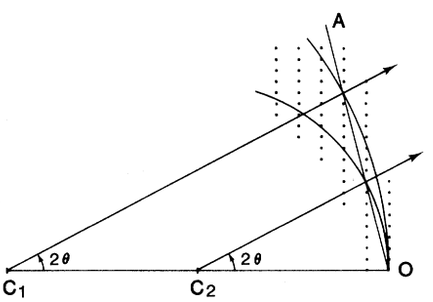

Fig. 1. The Ewald construction for Laue diffraction. A central section of the reciprocal lattice, origin O, is illuminated by x-rays of wavelength λ, where $\lambda_1 < \lambda < \lambda_2$. Diffraction from two reciprocal lattice points on the central lattice line OA occurs at the same scattering angle 2θ. Diffraction from other reciprocal lattice points not on this line occurs at different values of 2θ. $C_1O = 1/\lambda_1$ and $C_2O = 1/\lambda_2$.

Conventional diffraction experiments involving synchrotron radiation have used monochromatic radiation, in which a narrow bandpass, single-crystal monochromator with $\Delta\lambda/\lambda$ of the order of 10^{-4} selects a small portion of the continuous synchrotron x-ray spectrum. Monochromatization is generally believed (*2*) to be essential in preventing superposition of multiple orders of reflections, but our results show that this is not necessarily so.

The principle of Laue diffraction is illustrated in the Ewald construction (*6*) of Fig. 1. If polychromatic x-rays of wavelength λ, where λ lies in the range from λ_1 to λ_2 and $\Delta\lambda = \lambda_2 - \lambda_1$, fall on a crystal at point O, then all reciprocal lattice points that lie between the limiting Ewald spheres of radii $1/\lambda_1$ and $1/\lambda_2$ will be in diffracting position for some incident wavelength λ and will contribute to a Laue reflection. For all points on a radial reciprocal lattice line such as OA, diffraction will occur at a single value of 2θ; that is, the Laue reflection is multiple, with contributions from several structure factors.

Consider, for example, a reciprocal lattice point $(h'k'l')$ where $h' = nh$, $k' = nk$, and $l' = nl$; that is, $(h'k'l')$ is the nth order of (hkl). Then it may be shown that the condition for a Laue reflection to arise from a single structure factor $\mathbf{F}(h'k'l')$ is $n\lambda \leqq \lambda_1\lambda_2/\Delta\lambda$, where λ is the wavelength at which $(h'k'l')$ is in diffracting position. For small $\Delta\lambda$, this condition is approximated by $n \leqq \lambda/\Delta\lambda$. The fraction of lattice points that is of the nth order varies only slightly with the volume of reciprocal space included (determined by unit cell size, the resolution of crystal diffraction, and λ_1 and λ_2). Approximately 80 percent of all points are first order, and 98 percent are fifth order or less. With a bandpass $\Delta\lambda/\lambda$ of about 0.2, $\lambda/\Delta\lambda = 5$, and up to fifth order reflections will arise from only a single structure factor. Superposition of multiple orders of a reflection is therefore not a problem.

Laue diffraction from a stationary crystal results in an intensity whose integration is carried out over wavelength (*6*) rather than angle. Thus, rotation of the crystal is not required to record integrated intensities as it is in conventional oscillation and precession photography or single crystal diffractometry. By