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Electrophoretic Patterns of Hemoglobin from Fetal Mice of Different Inbred Strains

Abstract. *Starch gel electrophoretic patterns of hemoglobins from fetal mice of seven inbred strains (two with single, five with diffuse adult hemoglobins), were compared with each other and with electrophoretic patterns of hemoglobins from adults of the same strains. The blood from 15-day-old fetuses of all strains contained four electrophoretically separable heme components. However, there seems to be a difference between strains with single and diffuse adult hemoglobin in the time of emergence of a clear adult pattern.*

Two electrophoretically distinct types of hemoglobin are known in adult mice of different inbred strains (*1*). Electrophoretic studies with various supporting media have served to demonstrate inherited strain specificity for either the single (discrete single band) or the diffuse (two or more bands) type of hemoglobin (*2*). The difference between these patterns is controlled by a single pair of genes (Hb^s, single; Hb^d, diffuse). Recently, two reports have been published which describe the electrophoretic nature of mouse fetal hemoglobin (*3*, *4*). Additional components that contained heme, which are presumed to be fetal hemoglobin, were noted in early fetal mice of both the C57BL/6J strain (Hb^sHb^s) and the CBA strain (Hb^dHb^d). The concentration of these additional hemoglobins decreased steadily throughout fetal life and they were entirely absent at birth. Since the studies with these two inbred strains were carried out separately, no effort was made to compare the hemoglobins from Hb^sHb^s and Hb^dHb^d fetuses. The present experiments were conducted to determine whether or not electrophoretic differences can be detected when fetal hemoglobins from strains with single and diffuse adult hemoglobins are run side by side on a starch gel.

Mice from two Hb^sHb^s strains (C57BL/6J, SEC/Re) and five Hb^dHb^d strains (FL/Re, AKR/J, 129/J, DBA/2J, and CBA/J) were used. Data were collected from a minimum of five gels per strain and, in most cases, fetal hemoglobins from two or more Hb^dHb^d strains were placed on the same gel, together with their adult controls and a C57BL/6 sample of the same age.

Fetuses aged 15 days (from the day on which vaginal plugs were observed) were selected for use in screening the different strains because, at this stage, one litter was generally sufficient to ensure the collection of enough cells for hemoglobin testing while fetal components were still in evidence. The collected cells (*4*), usually 0.05 to 0.1 ml, were washed once by suspension in 10 ml of cold saline and sedimented in a clinical centrifuge at high speed for 15 minutes. The supernatant was drawn off and the cells were lysed in three times their volume of distilled water. The precipitation of stroma was effected by the addition of a salt solution (1.0*M* NaCl, 0.07*M* $MgCl_2$) in an amount equaling 15 percent of the total volume of lysate. Precipitated stroma were sedimented in a Servall Superspeed Centrifuge for 30 minutes at 15,000 rev/min, and the clear hemoglobin solutions decanted and converted to carboxyhemoglobin. All procedures, except the addition of CO, were carried out in the cold, and solutions were placed on a gel within 24 hours of sample collection. Separations were accomplished by starch gel electrophoresis using the discontinuous buffer system of Poulik (*5*), run for 2½ to 3 hours at approximately 5 volt/cm. Following the completion of a run, the gel was sliced and stained with Amido Black 10B (see *6*) or, less frequently, with 3,3′-dimethoxybenzidine.

When prepared by these methods, solutions from adults with single hemoglobin give a single band while those from adults with diffuse hemoglobin give two separable bands of unequal concentration with the highest concentration in the more rapidly moving component. Two additional characteristics of Hb^dHb^d hemoglobins (both fetal and adult) in these preparations are a tendency to streak in the medium and the appearance of an area projecting ahead of the major component which does not resolve to give a discrete band (Fig. 1). The number and mobilities of fetal components appear to be similar in preparations of Hb^dHb^d and Hb^sHb^s fetuses (Fig. 1). Fetuses of Hb^sHb^s strains produced a pattern consisting of four bands with a very high concentration in the most rapidly moving band, giving the hemoglobin pattern of these fetuses at 15 days' gestation an appearance close to that of an adult. The concentration of fetal components varied from litter to litter in all Hb^dHb^d strains tested. However, there was not the great resemblance to the adult pattern frequently observed in 15-day Hb^sHb^s fetal preparations. Fetal preparations from Hb^dHb^d strains showed a single band in the region of the adult major component, a second band in the region of the adult minor component, and two additional bands, which resembled the third and fourth components of Hb^sHb^s fetuses in their mobilities.

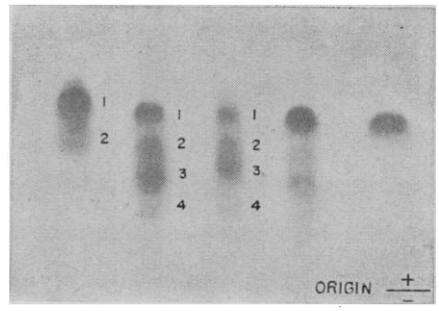

Fig. 1. Fetal and adult hemoglobin components on starch gel developed by staining with Amido Black 10B. Additional protein bands (*6*) which are also developed with this stain are not visible in this photograph. Left to right: AKR (adult), C57BL/6 (from 13-day fetus), AKR (14-day fetus), C57BL/6 (14-day fetus), C57BL/6 (adult).

To test the reality of apparent differences in maturity between the Hb^dHb^d and Hb^sHb^s fetal hemoglobin patterns, preparations from 14-day fetuses of one of the Hb^dHb^d strains (AKR) were placed on gels together with preparations from 13-day and 14-day fetuses of one of the Hb^sHb^s strains (C57BL/6). The results are shown in Fig. 1. The bands have been numbered for reference, beginning with the one nearest the anode. In AKR preparations it could be noted that band 3 had the highest concentration at 14 days. Bands 1 and 2 appeared to be in approximately equal concentration with respect to one another. In 14-day

C57BL/6 preparations band 1 was the most concentrated and band 3 the next highest in concentration. It was evident that in these 14-day Hb^dHb^d preparations band 3 was the major band, while in the Hb^sHb^s hemoglobin of the same age, band 1 was predominant. In Hb^dHd^d fetuses aged 15 days, band 1 (presumably the adult major band) in some, but not all, preparations seemed to have increased in concentration, taking up more stain than band 2; band 3 was still present in relatively high concentration. On the other hand, 15-day Hb^sHb^s fetuses showed a progressive increase in band 1 and a corresponding decrease in all other bands.

It seems reasonable to assume from these data that the development of an adult hemoglobin pattern is delayed in mice with the gene Hb^d; however, the possibility that different strain backgrounds, the presence of the additional adult hemoglobin in such mice, or both, may contribute to this appearance cannot be ignored. Detailed studies of the developing blood pictures of C57BL/6 and AKR/J fetuses are currently in progress. Crossing of the gene for diffuse hemoglobin into a single hemoglobin strain should give the two types of hemoglobin on a similar background and provide interesting information about the effects of background on fetal hemoglobin pattern (7).

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2. Analyses of hemoglobins from adult mice by column chromatography, chain separation, and peptide analysis have demonstrated that mice with the gene Hb^d have two hemoglobins in unequal concentrations (approximately 80:20). These differ from each other in the nature of their β-chains [J. J. Hutton, J. Bishop, R. Schweet, E. S. Russell, *Proc. Natl. Acad. Sci. U. S.* **48**, 1505 (1962)].
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6. Several additional bands, presumably protein contaminants, were developed by Amido Black staining of the gels. These were found in all of the mice examined. It was also interesting that a very fast band was present, moving ahead of the hemoglobin components. This component totally rejected the stain and thus appeared as a white spot against the blue background of the stained gel; it was observed in all fetal preparations but was not present in adults.
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New Natural Growth Promoting Substance in Young Citrus Fruit

Abstract. *A naturally occurring compound that induces curvature in the* Avena *coleoptile test has been found in young orange fruits. Cochromatography with C^{14}-labeled indole-3-acetic acid, plus excitation and fluorescence spectra determinations, indicates that this compound is not one of the known indoles or gibberellins.*

A compound which induces curvature of *Avena* coleoptiles but does not appear to be an indole compound has been extracted from young orange fruits. Since this material, which we have called "citrus auxin," does not seem to fit under any of the classes of known endogenous growth regulators: indoles, gibberellins, or phytokinins, it is possible that it may represent a new class of natural growth regulators.

For many years indole derivatives were believed to be the only naturally occurring growth promoters in higher plants. The first description of the occurrence of a non-indolic, gibberellin-like substance in higher plants was reported by Mitchell *et al.* in 1951 (*1*). Since then a number of reports relating to gibberellins or gibberellin-like substances have been published. Crosby and Vlitos (*2*) and Stowe (*3*) reported the existence of non-indolic growth-promoting substances. The first non-indolic growth-promoting substance to be isolated from a citrus tissue was gibberellin A_1 from the water sprouts of *Citrus unshiu* in 1959 (*4*). However, the gibberellins do not cause curvature of the *Avena* coleoptile. In fact, the only compounds which have been known to give a positive reaction in this classical assay for plant growth stimulators have been indole derivatives (*5*).

Peroxide-free ether, saturated with water, was used to extract the "citrus auxin" from lyophilized tissue of Valencia and Navel orange fruits about 10 mm in diameter. A 2-hour extraction was carried out in the dark at room temperature with constant shaking. Extraneous material such as lipids, pigments, and non-acidic compounds was removed from the extract by repeated acetonitrile-hexane and sodium bicarbonate-ether fractionations. By adjusting the *p*H of the bicarbonate fraction to 2.8 before ether extraction, the plant constituents extracted were the acidic compounds.

The concentrated ether extract finally obtained was then fractionated by two-dimensional descending paper chromatography with *n*-butanol-ammonia-water (4:1:1 vol/vol) and isopropanol-ammonia-water (10:1:1 vol/vol). All the extracting, concentrating, and analyzing by chromatography was conducted in the dark, either under a nitrogen atmosphere or in a vacuum.

Fluorescent and absorbing spots were detected by scanning the chromatogram with ultraviolet light (253 mμ, maximal intensity). These spots were eluted in water and their excitation and fluorescence spectra were determined on an Aminco-Bowman Spectrophotofluorometer. Cochromatography with C^{14}-labeled indole-3-acetic acid (IAA) was used to aid chromatographic identification. The *Avena* coleoptile curvature test (*6*) was used as a biological assay of the extracted substances.

The first biological assays indicated that there was a growth-promoting substance in the extract which chromatographed similarly with indole-3-acetic acid. This was the expected observation and one which has been observed in the extracts of many fruits. Upon subjecting this particular material to fluorometric characterization it was observed that it did not have the same fluorometric characteristics as indole-3-acetic acid. The wavelength at which maximum excitation of indole-3-acetic acid occurs is 290 mμ, and its maximum fluorescence wavelength is 360 mμ; "citrus auxin" has a maximum excitation wavelength of 350 mμ and a maximum fluorescence wavelength of

Table 1. Excitation and fluorescent characteristics of growth-regulating substances and related compounds.

Compound	Excitation wavelength (mμ)	Fluorescence wavelength (mμ)
Indole-3-acetic acid	290	360
Indole-3-pyruvic acid	290	350
Indole-3-propionic acid	300	365
Indole-3-butyric acid	290	365
Indole	295	365
α-Naphthaleneacetic acid	310	340
β-Naphthoxyacetic acid	330	350
Gibberellic and gibberellenic acids*	405	450–480
Kinetin	335	410
Reduced pyridine nucleotides	340	457
α-Naphthol	335	455
β-Naphthol	350	460
Citrus auxin	350	460

* Dissolved in 85-percent sulfuric acid.