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Absorption spectra of "Alpha Pigment" isolated from cecal contents of germ-free animals

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A STUDY of cecal contents of germ-free rats and mice carried out in this laboratory has led to the isolation of a pigment fraction ("alpha pigment") which exerted vasodilator effects and proved a strong stimulant to the spontaneous rhythmic contraction of intestinal villi.[1] A similar fraction obtained from the cecum of conventional control animals was found inert by the same criteria. In its biologic effects,[1] germ-free alpha pigment showed a close similarity to ferritin in its vasodepressor form as described by Mazur and Shorr.[2] Ferritin, the carrier protein of iron transport within the intestinal epithelium (and its iron-free form apoferritin), is known to reach the gut lumen in considerable quantity by the desquamation of intestinal epithelial cells (Crosby[3] and Conrad *et al.*[4]). It has been theorized that in the intestinal lumen ferritin and apoferritin are broken down by digestive processes to "subunits" (Harrison[5]) and to peptide complexes which form the basis for alpha pigment. Preliminary observations made by the gel filtration method have indicated that the molecular weight of alpha pigment is much smaller than that of ferritin or apoferritin. Under conditions prevailing in the germ-free host, alpha pigment retains the bioactive properties of ferritin. In the gut of conventional animals, the microflora appear to be responsible for the inactivation of alpha pigment. This was also suggested by observations *in vitro*, which showed that after trypsinization germ-free alpha pigment, ferritin and apoferritin could be inactivated by incubation with an inoculum of the intestinal flora.[1]

The purpose of this study was to observe absorption spectra of these substances as an extension of the work done on their biologic characteristics. Specifically, it aimed to consider light absorption: (1) of germ-free alpha pigment in comparison with those of ferritin and apoferritin in native and in trypsin-digested form, and (2) of alpha pigment, ferritin and apoferritin after microbial neutralization of their bioactive properties.

METHODS

The alpha pigment fractions used in this work originated from cecal contents of young adult germ-free and conventional rats and mice fed steam-sterilized L-462 diet[6] and from similar mice which 3 weeks prior to sacrifice, were switched from L-462 to the liquid amino acid-type diet of Greenstein *et al.*,[7] which was sterilized by filtration. The diets were obtained from a commercial source (General Biochemicals, Inc., Chagrin Falls, Ohio, GBI). In all details of animal holding, harvesting of cecal contents and preparation of alpha pigment fractions, the routines given in the previous publication were followed. The solvent generally used in this work was a modified Krebs phosphate buffer at pH 7·2,[1] unless differently stated.

Horse spleen ferritin and apoferritin were used (twice crystallized, Cd-free; GBI). The standard pigment reference was a ferritin solution containing 50 μg protein/ml. Since alpha pigments were insufficiently characterized by chemical criteria, they were spectrophotometrically matched to the ferritin standard at 420 mμ. The apoferritin solution was of the same concentration as that of ferritin.

The digestion of native ferritin and apoferritin was carried out by precipitation with ammonium sulfate, treatment with 8 N urea and by successive trypsinization according to the method of Harrison.[8]

The microbial inactivation of germ-free alpha pigment, ferritin and apoferritin was carried out by trypsin pretreatment (for 12 hr at 37° and in Krebs phosphate at pH 8·4; enzyme: substrate ratio 1:20) followed by incubation with a flora inoculum obtained from conventional rat cecum (for 48 hr at the same temperature and pH 7·2). Terminally, the incubate was centrifuged at 24,000 *g* for 30 min.

The absorption spectra were read in a Zeiss PMQ II spectrophotometer with 2-ml quartz cuvettes of 1 cm light path. When taking the readings, blanks were used which contained all the ingredients

of the reaction mixtures, except alpha pigment, ferritin or apoferritin. For each substance, 2–12 batches were prepared and generally tested in triplicate.

RESULTS

Fig. 1 illustrates absorption curves of germ-free alpha pigment (rat, L-462), native ferritin and apoferritin. In all three cases a plateau was indicated, approximately between 250 and 300 mμ. Some discrepancy was present in the exact location of the "shoulder". Approaching longer wavelengths, the optical density of alpha pigment was seen falling off more rapidly than in the case of ferritin or apoferritin.

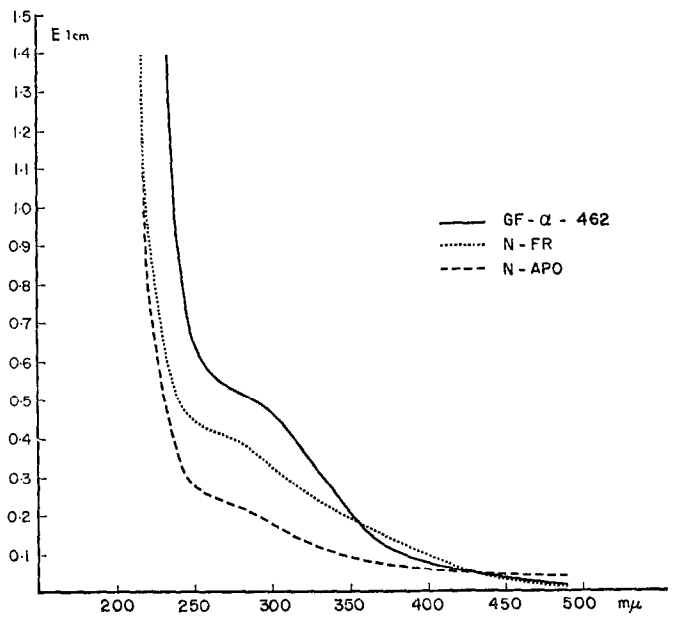

FIG. 1. GF-α-462 = germ-free alpha pigment (rat, L-462 diet); N-FR = native ferritin; N-APO = native apoferritin.

Fig. 2 shows the absorption of ferritin and apoferritin after treatment with trypsin according to the method of Harrison,[8] along with germ-free alpha pigment (mouse, liquid diet). Here, better alignment of the curves is indicated. The trypsin-treated samples of ferritin and apoferritin were tested in collaboration with Dr. E. Kokas on the intestinal villus preparation[1] and found fully active, along with the germ-free alpha pigment shown in Fig. 2.

Fig. 3 illustrates the absorption curves of trypsinized, flora-incubated and biologically inactive germ-free alpha pigment and ferritin, together with alpha pigment, similarly inactive, derived from conventional control animals. Considerable similarity in the plateau and in the "shoulder" of the curves is apparent in this figure.

These results suggest the following trends. (1) Alpha pigment from cecal contents of germ-free animals fed L-462 diet along with the ferritin (or apoferritin), which in their biologic characteristics showed virtual identity in the previous experimental series,[1] displayed only partial similarity in their absorption spectra. It is possible that this difference between alpha pigment and ferritin was caused by the heterogeneity of origin of these compounds. It is also conceivable that the effects of steam

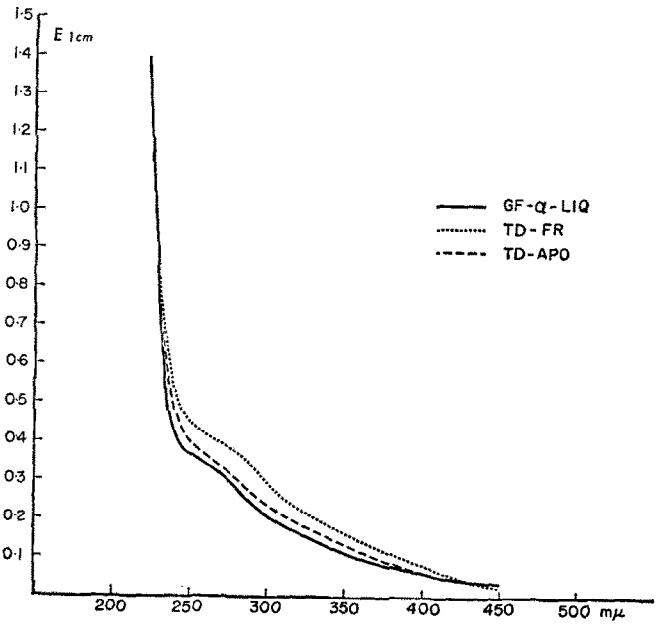

FIG. 2. GF-α-LIQ = germ-free alpha pigment (mouse, liquid diet); TD-FR = trypsin-digested ferritin; TD-APO = trypsin-digested apoferritin.

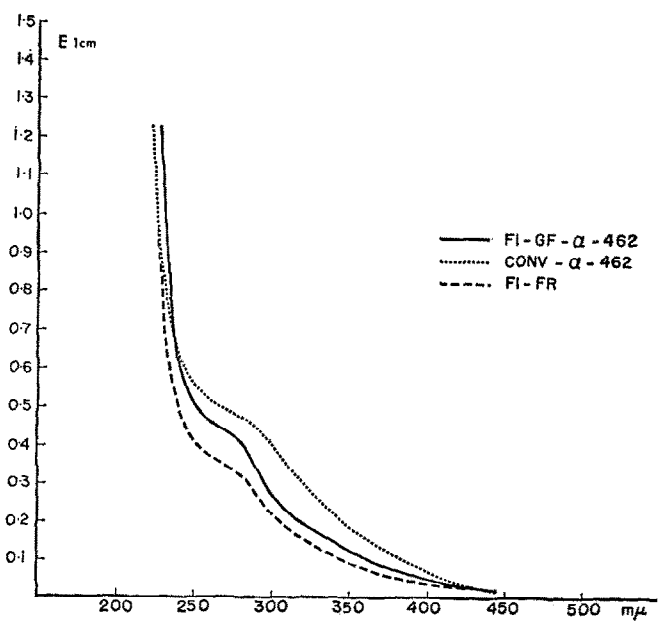

FIG. 3. FI-GF-α-462 = flora-incubated germ-free alpha pigment (rat, L-462 diet); CONV-α-462 = conventional alpha pigment (rat, L-462 diet); FI-FR = flora-incubated ferritin.

sterilization and the impurities of the practical-type L-462 diet were reflected in the germ-free gut contents. This speculation is supported by the closer spectral alignment between ferritin and alpha pigment derived from germ-free mice fed the filtration-sterilized, synthetic liquid diet, which is currently our purest source of biologically active alpha pigment.

(2) Ferritin and apoferritin, whether in native or in trypsin-treated form, showed considerable similarity in their absorption spectra. Digestion of ferritin and apoferritin by trypsin *in vitro* did not influence their bioactive properties on intestinal villus motility, as tested in the previous experimental series.[1] These observations suggest that ferritin contained in the intestinal epithelium, reaching the intestinal lumen on desquamation, may retain its spectral characteristics and bioactivity after exposure to intestinal digestion in the absence of bacteria. Alpha pigment originating from the germ-free gut, where trypsin and other digestive enzymes have been found even at higher levels than in conventional animals,[9, 10] displaying similar absorption, bioactivity and perhaps falling in the same molecular weight range as digested ferritin, may offer a counterpart *in vivo* to this speculation.

(3) Incubation of germ-free alpha pigment, ferritin and apoferritin with the intestinal microflora neutralizing the bioactive properties of these substances did not particularly alter their absorption spectra. A similar spectrum was obtained from alpha pigment derived from conventional control animals. This indicates that alpha pigment, ferritin and apoferritin were all sensitive to microbial inactivation. It is clear that their absorption characteristics do not indicate whether or not they are biologically active.

(4) In general, our observations on the absorption spectra of ferritin and apoferritin coincide with comparable data from the literature.[11, 12] In the present instance the consistent absorption in the u.v. range of these substances (though not very diversified or specific) appears to be a useful tool for probing their qualitative relationship to alpha pigment. The lack of particular differences in the spectra of such heterogeneous agents as horse spleen ferritin and rat gut alpha pigment, in addition to their bioactive similarity, may be taken as an indication of their related nature.

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Department of Pharmacology,
College of Medicine,
University of Kentucky, Lexington, Ky., U.S.A.

J. MAGYARY-KOSSA*
H. A. GORDON

* On leave of absence from J. R. Geigy, S.A., Basel.

REFERENCES

1. H. A. GORDON and E. KOKAS, *Biochem. Pharmac.* **17**, MS. 2113 (1968).
2. A. MAZUR and E. SHORR, *J. biol. Chem.* **176**, 771 (1948).
3. W. H. CROSBY, *Blood* **22**, 441 (1963).
4. M. E. CONRAD, L. R. WEINTRAUB and W. H. CROSBY, *J. clin. Invest.* **43**, 963 (1964).
5. P. M. HARRISON, *J. molec. Biol.* **1**, 69 (1959).
6. B. S. WOSTMANN, *Ann. N.Y. Acad. Sci.* **78**, 175 (1959).
7. J. P. GREENSTEIN, S. M. BIRNBAUM, M. WINITZ and M. C. OTEY, *Archs. Biochem. Biophys.* **72**, 396 (1957).
8. P. M. HARRISON, *J. molec. Biol.* **4**, 239 (1962).
9. B. BORGSTRÖM, A. DAHLQUIST, B. E. GUSTAFSSON, G. LUNDH and J. MALMQUIST, *Proc. Soc. exp. Biol. Med.* **102**, 154 (1959).
10. B. S. REDDY and B. S. WOSTMANN, *Archs. Biochem. Biophys.* **113**, 609 (1966).
11. S. GRANICK, *Chem. Rev.* **38**, 349 (1945).
12. J. W. DRYSDALE and H. N. MUNRO, *Biochem. J.* **95**, 282 (1965).