Fe^{59}-Amino Acid Complexes: Are They Intermediates in Fe^{59} Absorption Across Intestinal Mucosa?* (30409)

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Prior studies(1-3) demonstrate an active transport mechanism for iron absorption in rat duodenum *in vitro*. When Brown and Rother(4) reported the occurrence of Fe^{59}-amino acid complexes during transit of Fe^{59} across rat intestinal mucosa *in vivo*, it became of interest to determine whether such complexes were intermediates in the active transport. Repetition of the experiments of Brown and Rother confirmed their chromatographic evidence for these complexes, but inasmuch as the amounts recovered were very variable, each step in the authors' procedure was examined. As shown below, the chromatographic spots used to identify Fe^{59}-amino acid complexes result only if the mucosa is washed initially or treated subsequently with Versene-Fe^3 Specific.§ Moreover, chelates of Fe^{59} with Versene-Fe^3 Specific or ethylenediaminetetraacetate (EDTA) migrate on paper chromatography or electrophoresis at the same rates as the supposed Fe^{59}-amino acid complexes.

Methods. Male, albino, Sherman strain rats weighing 100-250 g were fasted overnight in metabolism cages without access to feces. The procedure of Brown and Rother (4) was then followed throughout. Rats were dosed by gastric tube with 20 μg of iron as $FeSO_4 \cdot 7H_2O$ and approximately 10 μc of $Fe^{59}SO_4$ (Abbott Laboratories, 24 mc/mg), and subsequently the proximal 20 cm of the small intestine was washed with isotonic saline and 0.5 M Versene-Fe^3 Specific(4), dissected open, the mucosa removed and homogenized in 10 volumes of 0.01 M sodium phosphate of pH 7.2. The non-particulate supernatant after centrifugation at 105,000 $\times$ *g* for 60 minutes in a model L Spinco ultracentrifuge was treated with perchloric acid (to a final concentration of 2%) to precipitate proteins, and the perchloric supernatant neutralized with 2 N KOH. The resulting protein-free solutions were concentrated by evaporation under vacuum and tested by paper chromatography or electrophoresis. In some experiments protein-free ultrafiltrates of the non-particulate supernatant solutions were prepared using a collodion bag suction apparatus (C. Schleicher & Schuell Co., Keene, N. H.).

Paper electrophoresis was performed initially as previously described(4). Similar results were obtained subsequently by a more convenient procedure, electrophoresis at 5°C between glass plates on Whatman 3 MM paper 19 cm wide for 3 hours at 350 V in 0.05 M sodium acetate of pH 4.8. Ascending paper chromatography was performed as described by Brown and Rother on either Reeve-Angel WA-2 amberlite-impregnated paper (developed in succinate buffer pH 6) or Whatman No. 1 with ethanol/cyclohexane/H_2O as solvent. Proportions of the latter solvent were modified to ethanol (210)/cyclohexane (10)/H_2O (to saturate) when the authors' proportions were found to yield an immiscible mixture. Paper strips were counted in a well-type scintillation counter and stained with 0.25% ninhydrin in acetone.

Results and discussion. Initial experiments included the Versene-Fe^3 Specific wash of the mucosa and yielded Fe^{59}-containing, ninhydrin positive areas with mobilities on paper electrophoresis and ascending chromatography similar to those described by Brown and Rother. Thereafter 5 experiments with individually dosed rats were performed without the Versene. Table I (a) shows that these same areas now contained no Fe^{59}, although

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§ Obtained from Dow Chemical Co.

TABLE I. Migration of Fe^{59}-Containing Material Obtained in Neutralized, Perchlorate Supernatant Solutions of Non-particulate Fractions of Mucosal Homogenate.*

Preparation	Paper electrophoresis Mobility cm	Paper electrophoresis % Fe^{59} recovered†	Paper chromatography Whatman No. 1 Rf	Paper chromatography Whatman No. 1 % Fe^{59} recovered	Paper chromatography amberlite-impregnated Rf	Paper chromatography amberlite-impregnated % Fe^{59} recovered
(a) Homogenate	0	0	0	0	0	0
(b) Homogenate + Versene-Fe^3 Specific	+7	33 ± 11	.46	68 ± 4	.75	87 ± 8
(c) Perchloric acid extract of (a) plus Versene-Fe^3 Specific	+9	59 ± 8	.48	41 ± 11	.85	96 ± 3
(d) Fe^{59} plus Versene-Fe^3 Specific	+8	95 ± 2	.62‡	92 ± 6	.78	79 ± 12
Fe^{59}	0		0			
Glycine	−3		.52		.90	

* Each of 5 male rats weighing approximately 150 g were anesthetized with ether and given 20 μg of iron as $FeSO_4 \cdot 7\ H_2O$ and Fe^{59} by direct needle puncture of the stomach wall and injection into the duodenal lumen. Eight minutes later the proximal 20 cm of small intestine were washed, excluding the Versene Fe-3 Specific wash, and the mucosa homogenized. A portion of homogenate (a) was fractionated directly, another aliquot (b) was treated with Versene Fe-3 Specific, pH 8.0, to a final concentration of 0.05 M prior to fractionation. Some of the neutralized perchlorate supernatant obtained from (a) was also treated with Versene Fe-3 Specific as above and is labelled (c) in the Table. The non-particulate fractions of the mucosal homogenates were treated with perchloric acid to precipitate proteins, neutralized with KOH, and the supernatant solutions tested by paper electrophoresis, or on Whatman No. 1 or amberlite-impregnated paper as described in *Methods*.

† Value indicates % of total Fe^{59} on the paper recovered in the specific area.

‡ Versene-Fe^{59} complex alone moved more rapidly than the corresponding areas in (a) and (b), but migrated identically when added to the perchlorate supernatant solutions of (a) or (b).

ninhydrin positive material persisted. Further, after addition of the Versene to whole homogenate (b), or to the perchlorate supernatant solutions (c), or to Fe^{59} alone, the radioisotope appeared again in areas corresponding to the apparent amino acid complexes.

When added to mucosal homogenates Versene-Fe^3 Specific and EDTA (which acted identically) also increased the non-particulate and non-protein bound fractions of Fe^{59}. Table II summarizes a representative experiment in which the proximal small intestine was washed with EDTA (20 ml of 0.5 M EDTA, pH 8.0) prior to the usual homogenization and fractionation procedure. Treatment with EDTA increased the non-particulate bound Fe^{59} by 135% and the ultrafiltrable Fe^{59} by 300%. Brown and Rother(4) noted that the value for non-protein bound Fe^{59} was similar as determined by either ultrafiltration or perchloric acid precipitation. As shown in Table II, these values agreed only after prior wash of the mucosa with EDTA. Without EDTA the non-protein Fe^{59} was 225% greater after perchloric acid precipitation as compared to ultrafiltration.

The foregoing results do not exclude the formation and participation of amino acid complexes in Fe^{59} transit across rat intestinal

TABLE II. Effects of EDTA and Perchloric Acid on Distribution of Fe^{59} in Mucosal Homogenates.*

Preparation	Fe^{59} non-particulate supernatant / Fe^{59} homogenate %	Fe^{59} ultrafiltrable / Fe^{59} non-particulate supernatant %	Fe^{59} perchloric supernatant / Fe^{59} non-particulate supernatant %
Homogenate, no EDTA wash	34	20	65
Homogenate, EDTA wash	80	80	85

* Two rats weighing 250 g were dosed with 20 μg iron as $FeSO_4$ and Fe^{59} as in Table I. The proximal 20 cm of small intestine were washed with EDTA (same conditions as for Versene Fe-3 Specific(4)) in one animal whereas this wash was eliminated for the other. Remaining fractionation procedure was as previously described(4).

mucosa. They indicate, however, that the chromatographic evidence provided thus far is inadequate to demonstrate such complexes and appears to result from the formation of Fe^{59}-Versene in the experimental procedure. Versene also alters the binding of Fe^{59} to mucosal particulates and both Versene and perchloric acid influence Fe^{59} binding to soluble proteins. Thus it seems necessary to avoid these reagents in procedures designed to study mucosal iron compounds of physiological significance.‖

‖ After preparation of this manuscript a report by Charlton *et al.*(5) described similar findings which indicate that the supposed Fe^{59}-amino acid complex is a complex of iron and Versene Fe-3 Specific.

Summary. Prior chromatographic evidence implicated the participation of Fe^{59}-amino acid complexes in intestinal absorption of Fe^{59}. Present studies show that the chromatographic evidence is inadequate to demonstrate such complexes and appears to result from the formation of Fe^{59}-Versene in the experimental procedure.

1. Dowdle, E. B., Schachter, D., Schenker, H., Am. J. Physiol., 1960, v198, 609.
2. Manis, J. G., Schachter, D., ibid., 1962, v203, 73.
3. ———, ibid., 1962, v203 81.
4. Brown, E. B., Rother, M. L., J. Lab. Clin. Med., 1963, v62, 357.
5. Charlton, R. W., Jacobs, P., Torrance, J. D., Bothwell, T. H., J. Clin. Invest., 1965, v44, 543.

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Hemadsorption and Immunofluorescence of Friend Virus in Cell Culture.* (30410)

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We previously reported propagation of Friend virus in mouse embryo cell culture (1). This article concerns an extension of the study with special reference to a hemadsorption reaction which was correlated with viral synthesis as revealed by immunofluorescence.

Materials and methods. The origin and method of preparation of Friend virus inoculum has been detailed elsewhere(1,2). The titer of a 10% infected spleen extract was approximately $10^{5.0}ID_{50}$/ml when inoculated intraperitoneally into Ha/ICR Swiss mice 4-5 weeks old obtained from the Roswell Park Memorial Institute breeding colonies.

The cell cultures used were secondary cultures of Ha/ICR Swiss mouse embryo tissues. Coverslip cultures were prepared using Eagle's basal medium containing 10% heat-inactivated calf serum as growth medium. Eagle's medium with 2.5% heat-inactivated horse serum was used as the maintenance medium.

The monolayer coverslip cultures were inoculated with $10^{5.0}$ mouse ID_{50}/ml of virus and incubated at 37°C for 2½ hours. After thorough washing, the cultures were incubated at 37°C overnight with growth medium followed by maintenance medium.

The indirect staining method was used in the immunofluorescent investigations(1,3). At appropriate intervals coverslips were removed from Leighton tubes, washed with phosphate buffered saline (PBS), dried at room temperature and then fixed in acetone for 10 minutes. Rabbit antiserum to Friend virus was placed on the coverslips and allowed to stand for 40 minutes at 37°C. This serum was previously absorbed twice with mouse liver powder, twice with normal spleen cells, and then twice with secondary mouse embryo cells. The coverslips were then rinsed 3 times with PBS and fluorescein isothiocyanate-conjugated anti-rabbit globulin antibody (Baltimore Biological Laboratory)

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