36, 365.
Oncley, J. L., Scatchard, G., and Brown, A. (1947), *J. Phys. Colloid Chem. 51*, 184.
Osuga, D. T., and Feeney, R. E. (1968), *Arch. Biochem. Biophys. 124*, 560.
Parker, W. C., and Bearn, A. G. (1962), *J. Exptl. Med 115*, 83.
Phelps, R. A., and Cann, J. R. (1956), *Arch. Biochem. Biophys. 61*, 51.
Rajagopalan, T. G., Moore, S., and Stein, W. H. (1966), *J. Biol. Chem. 241*, 4940.
Roberts, R. C., Makey, D. G., and Seal, U. S. (1966), *J. Biol. Chem. 241*, 4907.
Schachman, H. A. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic, p 75.
Schulman, S. (1953), *Arch. Biochem. Biophys. 44*, 230.
Spahr, P. F., and Edsall, J. T. (1964), *J. Biol. Chem. 239*, 850.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University, p 51.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, pp 365, 382.
Tanford, C., Kawahara, K., and Lapanje, S. (1967a), *J. Am. Chem. Soc. 89*, 729.
Tanford, C., Kawahara, K., Lapanje, S., Hooker, T. M., Jr., Zarlengo, M. H., Salahudoin, A., Aune, K. C., and Takagi, T. (1967b), *J. Am. Chem. Soc. 89*, 5023.
Van Holde, K. E. (1967), Fractions, No. 1, Beckman Instruments, Inc., p 1.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
Warner, R. C. (1953), *Trans. N. Y. Acad. Sci. 16*, 182.
Williams, J. (1962), *Biochem. J. 83*, 355.
Williams, R. C., and Rajagopalan, T. G. (1966), *J. Biol. Chem. 241*, 4951.
Windle, J. J., Wiersema, A. L., Clark, J. R., and Feeney, R. E. (1963), *Biochemistry 2*, 1341.

Isolation and Characterization of Rabbit Serum and Milk Transferrins. Evidence for Difference in Sialic Acid Content Only*

Erica Baker, D. C. Shaw, and E. H. Morgan

ABSTRACT: Transferrin has been isolated from rabbit serum by diethylaminoethyl Sephadex chromatography and electrophoresis. A similar iron-binding protein has been isolated from rabbit milk whey by electrophoresis and gel filtration. The two proteins are readily crystallized from distilled water at pH 5.3. Measurements have been made of nitrogen and iron content, molecular weight, light absorption spectra in the visible and ultraviolet ranges, and amino acid composition. The proteins have also been compared by electrophoresis on cellulose acetate and starch gel, by double diffusion in agar against specific antisera, and by two-dimensional peptide mapping of tryptic digests. The two proteins appear identical by all of these methods of analysis except electrophoresis, but this difference is eliminated by treatment with neuraminidase. The changes in mobility of the proteins after treatment with neuraminidase suggest that in serum most transferrin molecules contain two sialic acid residues while a few have only one residue; however in milk most molecules probably have one sialic acid residue, with a small proportion of the molecules having two. The molecular weight of the protein observed in the ultracentrifuge and on gel filtration calculates to be 70,000 daltons. The number of peptides observed on the maps, when considered in conjunction with the amino acid composition, suggests that this protein consists of two subunits.

Rabbit milk whey is remarkable for its extremely high iron-binding capacity due to the presence of a high concentration of an iron-binding protein with similar electrophoretic mobility to that of rabbit serum transferrin (Jordan *et al.*, 1967). In other species which have been studied iron-binding proteins are present in the milk in only low concentration and in the case of humans and the cow the major milk iron-binding protein is chemically and immunologically distinct from serum transferrin (Johansson, 1958, 1960; Montreuil and Mullet, 1960; Groves, 1960; Blanc and Isliker, 1961; Gorden *et al.*, 1962; Derechin and Johnson,

* From the Department of Physiology, The University of Western Australia, Nedlands, Western Australia, and the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory. *Received November 27, 1967.* This work was supported by grants from the Australian Research Grants Committee and the National Institutes of Health (5 RO5 TW-00212-02).

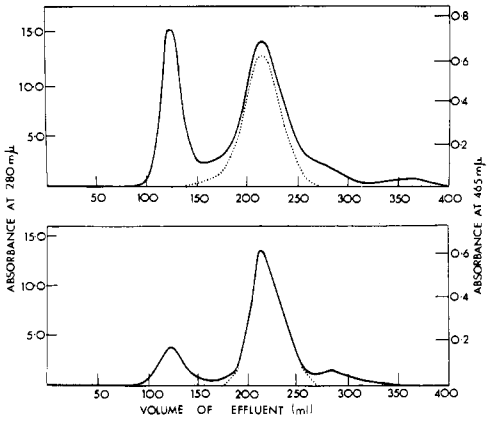

FIGURE 1: Sephadex G-200 gel filtration of rabbit whey to which iron had been added (above) and of the β-mobility fraction obtained from the whey by electrophoresis on Pevikon (below). Absorbance at 280 mμ is shown by the continuous lines and absorbance at 465 mμ by the dotted lines.

1962). It therefore seemed of considerable interest to purify rabbit milk iron-binding protein and compare it with rabbit serum transferrin. This paper reports the results of such a study. The properties of the milk protein were found to be so similar to those of serum transferrin that the milk protein will be referred to as milk transferrin.

Materials and Methods

Serum Transferrin. Rabbit serum transferrin was purified as described previously (Morgan, 1964) except that the chromatography step was performed using DEAE Sephadex A-50 and a buffer gradient from 0.05 M Tris-HCl (pH 8.5) to 0.2 M Tris-HCl (pH 7.5). Further purification, where necessary, was achieved by crystallization. Solutions of the protein were dialyzed for 2 days against four changes of distilled water and the pH was adjusted to 5.3 with 2% acetic acid. Crystallization usually commenced within a few hours and was complete within 48 hr when the solution was allowed to stand at 4°. It also occurred but proceeded more slowly at room temperature.

Milk Transferrin. Rabbit milk was collected manually, after the subcutaneous injection of 0.25 iu of oxytocin, and was centrifuged at 24,000*g* for 2 hr at 4°. The clear middle layer (whey) was removed and recentrifuged as before. Sufficient iron as ferrous ammonium sulfate was then added to saturate the transferrin of the clear whey. The whey was concentrated by ultrafiltration against barbiturate buffer (Laurell *et al.*, 1956) to one-half to one-third of its original volume. Electrophoresis was then performed using the same buffer and Pevikon (Superfosfat Fabriks, Stockholm, Sweden) as the anticonvectant material (Muller-Eberhard, 1960). The red, transferrin-containing band was eluted from the Pevikon in 0.15 M sodium chloride by centrifugation and concentrated by ultrafiltration. When examined by cellulose acetate electrophoresis the solution was found to contain only one protein band, but on starch gel electrophoresis this band was resolved into two bands close together in the transferrin region, and also a slower and a faster migrating component. The transferrin components were separated from the other two components by gel filtration on Sephadex G-200 (Figure 1). The pink, transferrin-containing fractions from the gel filtration column were concentrated by ultrafiltration. The transferrin was then usually free of the contaminant bands when examined by starch gel electrophoresis. However, if further purification was required this was easily achieved by crystallization using the same procedure as for serum transferrin.

Electrophoresis. Cellulose acetate electrophoresis was performed with Seprephore III strips (Gelman Instrument Co., Ann Arbor, Mich.) and barbitone–sodium acetate buffer (Brackenridge, 1960). Horizontal starch gel electrophoresis was performed using a discontinuous buffer system (Ashton and Braden, 1961).

The effect of neuraminidase on electrophoretic mobility of the transferrin preparations was determined by incubating the transferrins (0.5 mg) dissolved in 0.1 ml of 0.01 M calcium chloride–0.05 M sodium acetate buffer (pH 5.2) with 0.1 ml of neuraminidase (500 units/ml) isolated from *Vibrio cholerae* (Behringwerke, Marburg-Lahn, Germany). Samples were taken from the incubation solution after varying periods of incubation and examined by starch gel electrophoresis.

Gel filtration was used for the purification of milk transferrin and for the determination of molecular weight of the purified transferrins (Andrews, 1965). Sephadex G-200 suspended in 0.5 M sodium chloride–0.05 M Tris-HCl (pH 8.0) was packed into a 65 × 2.5 cm column as described by the manufacturers of Sephadex. The column was allowed to equilibrate with the buffer at a hydrostatic pressure of 100 cm of H_2O for 48 hr before use. The density of the solutions to be applied to the column was increased by adding sucrose to a concentration of 25%. They were then applied to the top of the gel beneath the buffer. Elution was performed using the buffer with which the gel was packed. Fractions of 10-ml volume were collected and their optical densities were measured at 280 mμ (and 465 mμ when transferrin was present). The column was calibrated with Blue Dextran (A. B. Pharmacia, Uppsala, Sweden) and cytochrome C, rabbit serum albumin, and rabbit γ-globulin assuming molecular weights of 13,200, 70,000, and 188,000, respectively, for the three proteins (Edsall, 1953; Charlwood, 1959a,b). For the purification of rabbit milk transferrin the samples were applied in a volume of 10 ml, but for molecular weight determinations a volume of 2 ml was used. All of the gel filtration procedures were performed in a cold room at 4 ± 2°.

Protein and Iron Estimations. Protein was measured by a biuret method (Kingsley, 1942), by nitrogen analysis, by the ninhydrin method (Jacobs, 1962), and by weighing freeze-dried samples of the purified transferrin. Iron was determined by the use of sulfonated bathophenanthroline as described for serum iron (Morgan and Carter, 1960).

Ultracentrifugal Analysis. The proteins were examined at concentrations of 3–15 mg/ml in 0.02 M Tris-HCl–0.145 M sodium chloride (pH 7.5) in a Spinco Model E analytical ultracentrifuge.

Spectral Analysis. Absorption spectra in the visible and ultraviolet regions were determined using a Beckman D. B. spectrophotometer. Visible spectra were examined in 0.07 M sodium phosphate buffer (pH 7.4) and ultraviolet spectra in 0.1 N hydrochloric acid, 0.07 M sodium phosphate buffer (pH 7.4), and 0.1 N sodium hydroxide. Extinction coefficients were determined at the wavelengths at which maximum absorption was obtained. All values correspond to extinction of a 1% protein solution in a 1-cm cuvet at 20°.

The effect of pH on the extinction at the wavelength of maximum absorption in the visible range (465 mμ) was studied by stepwise addition of 0.05 N hydrochloric acid to 1 ml of transferrin solutions (0.1 M sodium acetate buffer, pH 7.45 initially) followed by spectrophotometric readings after 20-min equilibration at room temperature. The curves so obtained were corrected for dilution using a control transferrin solution to which was added equivalent volumes of the starting buffer.

Immunological Methods. Guinea pigs were immunized with purified rabbit serum transferrin and milk transferrin, 2 mg of each protein in 0.2 ml of 0.15 M sodium chloride plus 0.2 ml of Freund's adjuvant being given intramuscularly three times at 2-week intervals. They were bled by heart puncture 2 weeks later. Immunodiffusion in agar was performed using 1.5% agar in 0.9% sodium chloride on microscope slides. Scheidegger's (1955) microslide technique was used for immunoelectrophoresis.

Amino Acid Analysis. The amino acid analyses were performed by the method of Spackman *et al.* (1958) on a Beckman Model 120B amino acid analyzer. Duplicate samples of the proteins were hydrolyzed in 6 N hydrochloric acid in sealed, evacuated tubes at 110° for 22 hr. As the purpose of the analyses was to compare the two proteins, the values were not corrected for decomposition of serine and threonine nor for incomplete hydrolysis. Separate determinations for cystine, as cysteic acid, were not performed. Therefore the values for this amino acid are only approximate.

The number of residues of each amino acid was calculated from the analytical results on the arbitrary basis of 24 residues of arginine. Summation of the product of these numbers multiplied by their respective residue weights gives a molecular weight of approximately 70,000 which value had been obtained by physical measurements.

Peptide Mapping. Performic acid oxidation was used to denature the proteins (Hirs, 1956). The oxidized proteins were hydrolyzed with trypsin (Worthington, lyophilized, two-times-crystallized lot TRL 6246) using 1% by weight of enzyme to protein. The hydrolyses for the initial comparisons were performed in 0.5% ammonium bicarbonate, volatile buffer, at 37° for 4 hr. Some later hydrolyses were performed without buffer using a Radiometer Model TTT 1 titrator to follow the course of hydrolysis, by recording the addition of alkali to maintain the pH at 8.0. In these cases it was found that hydrolysis was essentially complete after 15 min, there having been rapid addition of alkali during the first 10 min. The mixture was maintained at 37° for a further 60 min. If additional trypsin was added after the first 15 min no further burst of hydrolysis was observed.

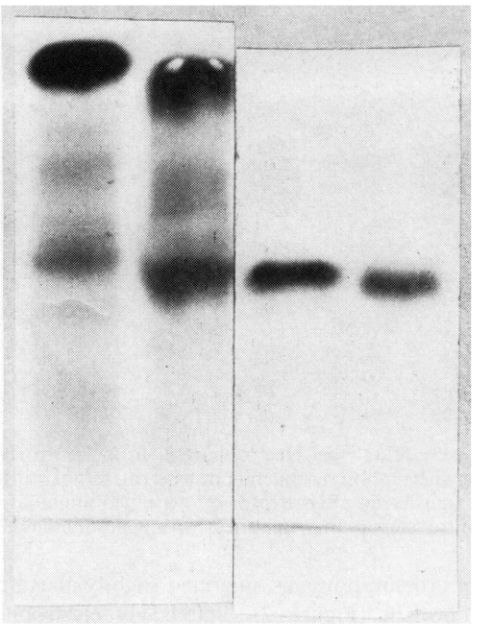

FIGURE 2: Cellulose acetate electrophoresis from left to right, of rabbit serum, rabbit whey, serum transferrin, and milk transferrin.

The hydrolysates in buffer were lyophilized, dissolved in water (10 mg/ml), and glacial acetic acid was added dropwise to give pH 4.0, when faint precipitation occured. These less soluble "core" peptides were separated by centrifugation. They accounted for less than 5% by weight of the original protein. The soluble peptides were lyophilized ready for peptide mapping. In some cases when hydrolysis had been followed by titration the whole mixture at pH 4.0 was lyophilized.

Peptide maps were prepared on Whatman No. 3MM paper. When hydrolysates were to be compared they were subjected to electrophoresis in parallel for the first dimension. High-voltage electrophoresis was performed in tanks designed after Michl (1959) using Varsol as heat exchanger. The strips containing the samples were cut out and sewn onto fresh sheets of paper for chromatography or electrophoresis at a different pH, at right angles to the initial separation. The two-dimensional maps were stained with ninhydrin, chlorine–starch–potassium iodide, and histidine-, tyrosine-, and arginine-specific stains (Easley, 1965).

Results

Electrophoresis and Effect of Neuraminidase. When examined by electrophoresis on cellulose acetate the serum and whey iron-binding proteins were found to migrate as single bands with electrophoretic mobilities corresponding to the β-globulin band of serum. The

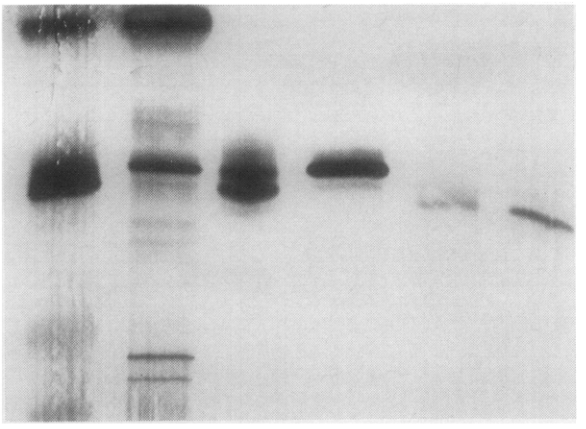

FIGURE 3: Starch gel electrophoresis from left to right, of rabbit whey, rabbit serum, milk transferrin, serum transferrin, milk transferrin after treatment with neuraminidase, and serum transferrin after treatment with neuraminidase.

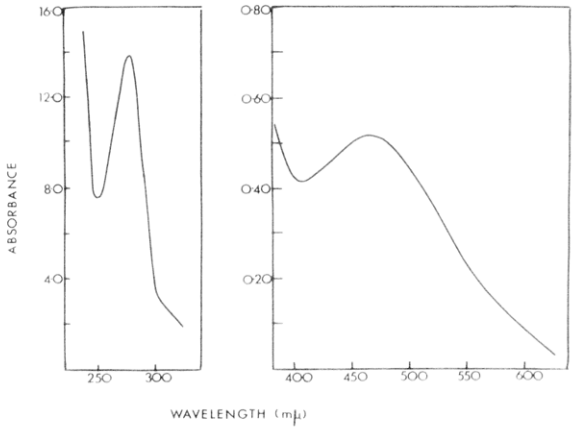

FIGURE 5: Spectrum of rabbit serum and milk transferrins in 0.07 M sodium phosphate buffer (pH 7.4). Since the spectra of the two proteins were the same only one is shown in the figure.

serum protein, however, migrated slightly ahead of the milk protein (Figure 2). Starch gel electrophoresis resolved the proteins each into two bands, the more prominent in the case of serum transferrin being the band of greater electrophoretic mobility, and with milk transferrin the band of slower mobility (Figure 3). Treatment of both proteins with neuraminidase for 20 hr reduced the electrophoretic mobility of the two bands to a single, slower moving band as far behind the original slow band as the latter was behind the fast band (Figure 3). After shorter periods of incubation with neuraminidase the two proteins were resolved into three bands corresponding to the two original bands and the newly formed slower moving band.

Crystals. No difference was found between the two proteins in the case of crystallization or in the form of the resulting crystals as seen under the microscope (Figure 4). The ease of crystallization after dialysis and adjustment of the pH to 5.3 was remarkable. Although no attempt was made to study this in detail, it was found that the transferrin could be crystallized from impure fractions which contained no more than 50–60% transferrin as indicated by cellulose acetate electrophoresis.

Nitrogen Content. Based on the weights of freeze-dried proteins the nitrogen contents were 14.7 and 14.9% for the serum and milk proteins, respectively.

Iron Content. The iron content of the preparations was found to be 1.42 and 1.45 μg of iron per mg of protein for the serum and milk proteins, respectively, based on measured weights of freeze-dried proteins.

Molecular Weight. The molecular weight as determined by gel filtration was 70,000 for both serum and milk proteins. The values obtained from sedimentation and diffusion analyses, assuming a partial specific volume of 0.725 (Oncley *et al.*, 1947), were 70,000 daltons for milk transferrin and 75,000 daltons for serum transferrin. The molecular weights calculated from the iron content of the freeze-dried proteins (which could contain about 10% moisture) assuming two iron atoms per protein molecule, were 77,000 and 79,000 for the serum and milk proteins, respectively.

Spectral Analysis. The spectra of the two proteins were found to be identical in both the visible and the ultraviolet range as long as heme compounds had been eliminated from the serum preparation by recrystallization (Figure 5). In the visible region maximum extinction was found at 465 mμ. In the ultraviolet region an absorption maximum was obtained at 279 mμ in neutral buffer, at 276 mμ in acid, and two peaks at 282 (trypto-

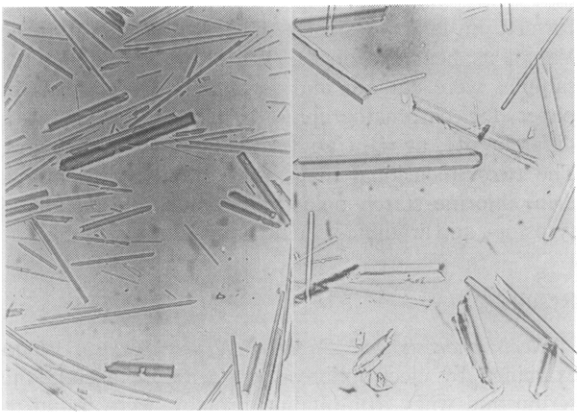

FIGURE 4: Crystals of rabbit serum transferrin (left) and milk transferrin (right), magnification 51×.

TABLE I: Extinction Coefficients of Rabbit Serum and Milk Transferrins at pH 7.4 in 0.07 M Sodium Phosphate Buffer.

Wavelength (mμ)	$E^{1\%}_{1\text{ cm}}$ Serum Transferrin	$E^{1\%}_{1\text{ cm}}$ Milk Transferrin
279	13.86	13.78
410	0.404	0.416
465	0.509	0.523
279–465	27.2	26.3
465–410	1.26	1.26

TABLE II: Amino Acid Composition of Rabbit Serum and Milk Transferrins.

	Residues/70,000 Mol Wt[a]		Residues/ 10,000 Mol Wt
	Serum	Milk	Serum
Lys	51.7	51.0	7.4
His	16.4	16.0	2.3
NH_3	61.2	59.6	8.7
Arg	24.0	24.0	3.4
Asp	70.2	70.1	10.0
Thr	20.3	20.4	2.9
Ser	33.6	33.5	4.8
Glu	60.9	60.7	8.7
Pro	32.5	32.8	4.6
Gly	45.1	44.6	6.4
Ala	51.2	49.5	7.4
Cys ($^1/_2$)	32.2	31.4	4.6
Val	42.3	42.0	6.0
Met.	5.6	5.4	0.8
Ileu	15.1	15.2	2.2
Leu	58.4	57.4	8.3
Tyr	23.2	22.8	3.3
Phe	24.7	24.2	3.5
Trp[b]	9.4	10.6	1.3

[a] The Values for amino acid residues for a molecular weight of 70,000 were calculated on the basis of assuming 24 residues of arginine (corresponding to a molecule of approximately 70,000). [b] Approximate value for tryptophan calculated from spectra.

phan) and 289 mμ (tyrosine) were obtained in alkali. The extinction coefficients at 465 and 279 mμ and the ratios of extinctions at 465:410 mμ and 179:465 mμ were very similar for the two proteins (Table I).

The relation found between optical density of the transferrin solutions at 465 mμ and pH is shown in Figure 6. The curves for the two proteins were the same. As the pH was lowered the optical density commenced to fall at pH 6.1, was 50% of its initial value at pH 4.6, and was zero at pH 4.0.

Immunological Analysis. No difference was found between the two proteins when they were examined by immunoelectrophoresis; on double diffusion in agar they showed reactions of identity (Figure 7).

Amino Acid Analysis. The amino acid compositions of the serum and milk proteins are presented in Table II. Any slight differences between the proteins are well within the experimental accuracy of the method.

Peptide Maps. Comparative peptide maps which were stained with ninhydrin are shown in Figure 8. In this and in other two-dimensional systems used for separation of the peptides, whether stained with ninhydrin, chlorine–starch–potassium iodide, or residue-specific reagents, there were no significant or consistent differences between the protein hydrolysates from the

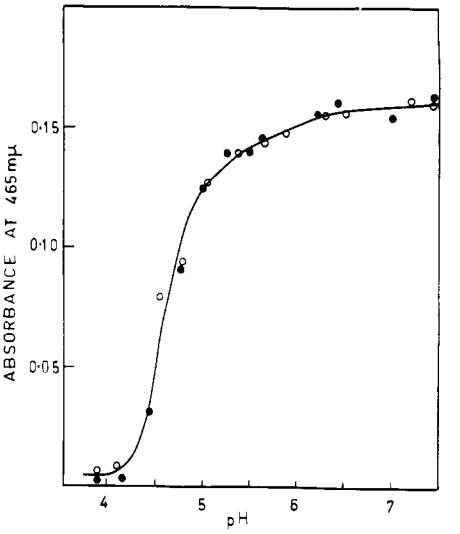

FIGURE 6: Effect of pH on absorbancy at 465 mμ of rabbit serum transferrin (open circles) and milk transferrin (closed circles).

two sources. The apparent difference in sialic acid content of two proteins did not cause any detectable change in mobility of the soluble peptides. Williams (1962) showed an identity between the peptides from chicken serum transferrin and conalbumin which differ in sialic acid content.

The number of peptides observed was less than expected, and so, in order to consider the possibility of chemical subunit structure of the transferrins, a peptide map obtained from the total digest (including "core") using a different two-dimensional separation was performed (Figure 9). The number of peptides counted in this and other maps is recorded in Table III.

Discussion

By all of the methods of analysis used in the present work except electrophoresis the serum and milk iron-

TABLE III: Number of Peptides Observed on Tryptic Maps of Rabbit Serum Transferrin.

	Number of Peptides			
		Maximum Expected for Min Mol Wt		
Stain	Obsd	70,000	35,000	45,000
Ninhydrin	53–58	77	39	45
Chlorine–starch–potassium iodide	47–51			
Pauly His	12–13	16	8	10
Pauly Tyr	12–14	23	12	14
α-Nitroso-β-naphthol (Tyr)	14–17	23	12	14
Sakaguchi (Arg)	14–16	24	12	14

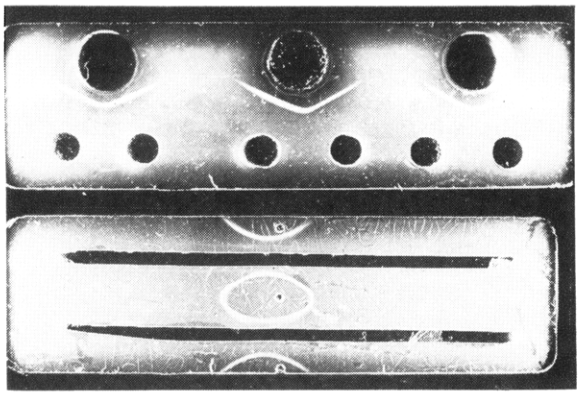

FIGURE 7: Immunoelectrophoresis (above) and double diffusion in agar (below) of rabbit serum and milk transferrins. For the immunoelectrophoresis, serum transferrin was placed in the center, milk transferrin on each side, antiserum transferrin in the upper trough, and antimilk transferrin in the lower trough. For the diffusion in agar, serum transferrin (center) and milk transferrin (each side) were each diffused against antimilk transferrin (left well) and antiserum transferrin (right well).

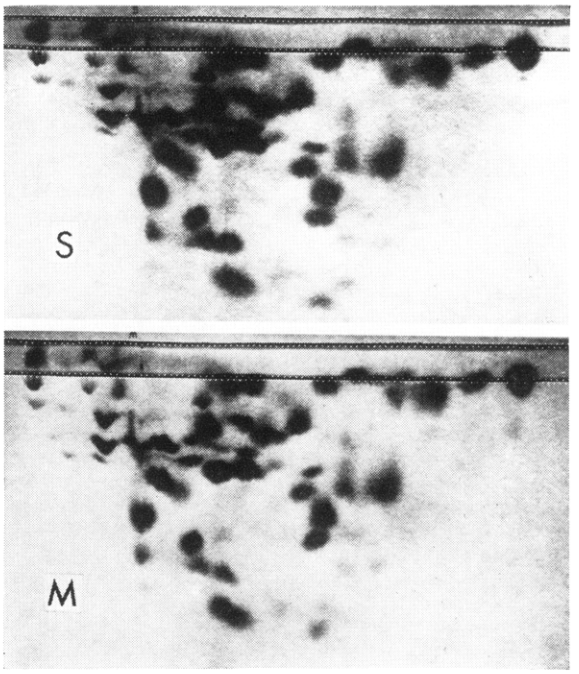

FIGURE 8: Two-dimensional peptide maps of soluble tryptic peptides from rabbit serum (S) and milk (M) transferrins, stained with ninhydrin. First dimension, pH 4.7 (pyridine, 25 ml, glacial acetic acid, 25 ml to 1 l. with water), 40 V/cm, 1 hr; second dimension, ascending chromatography isoamyl alcohol–pyridine–water (35:35:30, v/v).

binding proteins were found to have identical properties. It therefore seems justifiable to call both of the proteins transferrin. The experiments with neuraminidase indicate that the electrophoretic differences are probably due to differences in content of sialic acid. The fact that the two protein bands on starch gel could be reduced with neuraminidase to one of slower mobility than the first two suggests that some rabbit transferrin molecules contain two sialic acid residues and some contain one residue. In the case of serum transferrin the two sialic residue type of transferrin predominates while in milk the one residue type is present in higher concentration. The relationship between rabbit milk and serum transferrins is similar to that between conalbumin and chicken serum transferrin (Williams, 1962). These two iron-binding proteins were found to differ only in their carbohydrate prosthetic groups and the chicken serum transferrin showed changes on starch gel electrophoresis after neuraminidase treatment similar to those found in the present work.

The iron-binding proteins of whey have been studied in the milk of the human, cow, rat, and a marsupial, the quokka (*Setonix brachyurus*), as well as the rabbit. In the case of humans and cows two distinct proteins are present, one corresponding to serum transferrin, present in very low concentration, and one in higher concentration and with different properties to the serum protein (Schafer, 1951; Johansson, 1960; Montreuil and Mullet, 1960; Groves, 1960; Gordon *et al.*, 1962, 1963; Derechin and Johnson, 1962; Blanc *et al.*, 1963; Blanc and Isliker, 1964; Montreuil *et al.*, 1965). The latter protein has been called "red protein," "lactotransferrin," or "lactoferrin." It differs from serum transferrin immunologically, in amino acid composition, and in the effect of decreasing pH on the extinction at 465 mμ, when a fall in extinction does not occur until the pH reaches approximately 3. Differences in peptide maps have also been reported between the human serum and milk proteins (Spik and Montreuil, 1966). In contradistinction to the human and cow, the rabbit appears to have only one red milk iron-binding protein and this is extremely similar in properties to the serum protein. The method used to isolate the protein from rabbit whey was such that all red protein should have been detected. The milk iron-binding proteins of the rat and quokka differ from their serum counterparts in electrophoretic mobility on starch gel (Ezekiel *et al.*, 1963), but appear to be very similar immunologically (Laurell and Morgan, 1965; S. M. Jordan, personal communication). It seems possible that the relationship between milk and serum iron-binding proteins in these two species is similar to that in the rabbit. However the concentrations of the protein in their milk is very much less than with the rabbit (Ezekiel, 1965; S. M. Jordan, personal communication).

In many of its properties rabbit transferrin is similar to the transferrins of other species. It binds two atoms of iron per molecule with the development of a pink color and production of a light absorption spectrum in the visible range similar to that of human (Surgenor *et al.*, 1949), pig (Laurell, 1953), and rat (Gordon and Louis, 1963) transferrin. The change of extinction at 465 mμ with pH, and the crystal form, amino acid composition, and electrophoretic mobility are also similar to those of human transferrin (Surgenor *et al.*, 1949; Koechlin, 1952; Schultz *et al.*, 1958). Human transferrin, however, contains four sialic acid residues per mole (Schultze *et al.*, 1958; Parker and Bearn,

1962; Jamieson, 1965), compared with two residues for rabbit transferrin.

The source of rabbit milk transferrin could be either transfer from the plasma or synthesis within the mammary gland. It is possible that during transfer from plasma to milk sialic acid is removed from some of the transferrin molecules by the action of neuraminidase present in the mammary gland, leading to the change in starch gel electrophoretic mobility which was observed. Neuraminidase has been demonstrated in the mammary gland of the rat (Carubelli *et al.*, 1962). However, *de novo* synthesis within the gland appears to be the more likely source of milk transferrin. The amount present in the milk secreted each day, about 2 g (Jordan *et al.*, 1967), is about 20 times larger than the normal turnover in the plasma (Morgan and Finch, 1966). In addition Jordal (1963) and S. M. Jordan (personal communication) were unable to demonstrate any transfer of radioactive iodine-labeled transferrin from serum to milk in the rabbit. In this regard, also, the situation with the high concentration of transferrin in rabbit milk is similar to the high concentration of conalbumin in egg white where the iron-binding protein is apparently not derived from the blood plasma but is synthesized in the oviduct (Williams, 1962). Possibly both of these iron-binding proteins serve similar functions, to protect the chicken or suckling rabbit against pathogenic microorganisms. It has been demonstrated that the gut of the suckling rabbit is almost free of microorganisms (Smith, 1965). The transferrin in rabbit milk does not appear to aid iron transfer into the milk or to affect iron absorption by the suckling young (unpublished data).

The data from the ultracentrifuge and gel filtration experiments indicate a physical entity of molecular weight approximately 70,000. If this physical entity consisted of a single peptide chain, with nonrepeating amino acid sequence, then trypsin, which hydrolyses after each residue of lysine and arginine, should yield on the basis of the amino acid analysis about 77 peptides. In fact, even though the peptide spots on the maps prepared were apparently well resolved, the total number was in the range 47–58 spots.

The limitations of the method of peptide mapping as a means of estimating minimum molecular weight have been discussed by Harris and Hindley (1965). In the present case very little insoluble "core" material occurred and when this was included only a slight streaking from the origin was observed (Figure 9). Both ninhydrin and chlorine–starch–potassium iodide were used in detecting the peptides; these two methods of staining compliment one another, ninhydrin being most sensitive for short peptides while chlorine reacts with peptide bonds and so a darker spot is given with larger peptides. In all maps there were about ten peptides included in the count which in fact stained lightly with both reagents and therefore are more likely to be present in only low yield and due to limited extra cleavage caused by traces of chymotrypsin in the trypsin used, or double spotting due to amide or oxidation differences during preparation. The possibility remains, however, that the two-dimensional electrophoretic and

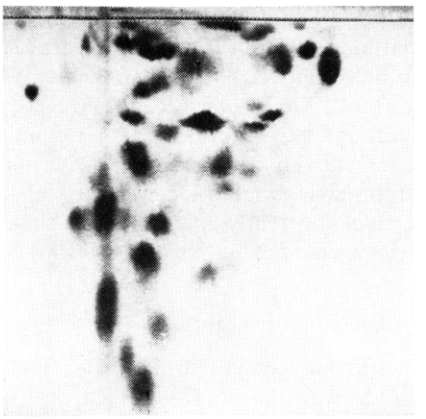

FIGURE 9: Two-dimensional peptide map of tryptic digest (including core) of rabbit serum transferrin, stained with ninhydrin. First dimension, pH 4.7 (as in Figure 8), 40 V/cm, 50 min; second dimension, descending chromatography butanol–acetic acid–water (3:1:1, v/v).

chromatographic systems would not be adequate for resolving an hydrolysate which did contain 80 peptides. This technical difficulty is largely overcome by running several portions of the hydrolysate in parallel in the first dimension and then subjecting each to a different chromatographic system in the second dimension. Examination of these maps on the basis of the number of spots observed for each centimeter along the first, common dimension did not indicate any region of unresolved overlapping in the systems shown in Figures 8 and 9. In addition the use of residue-specific stains can partly compensate for possible overlapping peptides as it is unlikely that the peptides containing a particular amino acid would always overlap one another. The limitation to this approach is that a peptide could contain more than one residue of the amino acid so that it is only possible to list the maximum number of peptides which could contain a particular amino acid in a digest of a protein of given molecular weight and amino acid composition. The specific stains are often more sensitive than ninhydrin and so any minor spots due to chymotryptic cleavage could give an appreciable color and have been included. Certainly in no case was the number of spots observed as high as that expected for a unit of 70,000 and yet it is more likely that more spots are produced by the technique than the number of unique tryptic peptides involved. The peptide maps have therefore been interpreted as indicating a subunit structure for transferrin; the 70,000 mol wt unit being composed of two similar if not identical chemical units, with a minimum molecular weight in the range 35,000–45,000.

Williams (1962) has published maps of tryptic peptides from chicken conalbumin and chicken serum transferrin showing 36 peptide spots plus a streak in the second dimension from the material neutral at pH 6.5; he did not comment on the possibility of chemical subunits but such a conclusion could be drawn from the maps, provided that a large amount of material had not remained at the origin and the streak did not contain more than 10–15 peptides. There is a brief

report (Jeppsson and Sjöquist, 1964) that the $s_{20,w}$ of reduced human serum transferrin in urea suggested a smaller molecular weight unit than the native protein. However, F. C. Greene and R. E. Feeney (personal communication) have not been able to obtain such evidence for chicken conalbumin. Two chemical subunits in transferrin would be consistent with the existence of two apparently identical iron-binding sites on the physical entity of mol wt 70,000–90,000.

Acknowledgment

The writers are grateful to Professor I. T. Oliver for assistance with the ultracentrifugal analyses. This work was performed during tenure of a CSIRO senior postgraduate studentship by E. Baker.

References

Andrews, P. (1965), *Biochem. J. 96*, 595.
Ashton, G. C., and Braden, A. W. N. (1961), *Australian J. Biol. Sci. 14*, 248.
Blanc, B., Biyard, E., and Mauron, J. (1963), *Experientia 19*, 299.
Blanc, B., and Isliker, H. (1961), *Bull. Soc. Chim. Biol. 43*, 929.
Blanc, B., and Isliker, H. (1964), *Bull. Soc. Chim. Biol. 46*, 963.
Brackenridge, C. J. (1960), *Anal. Chem. 32*, 1357.
Carubelli, R., Ryan, R. C., Trucco, R. E., and Caputto, R. (1962), *J. Biol. Chem. 236*, 2381.
Charlwood, P. A. (1959a), *Biochim. Biophys. Acta 32*, 283.
Charlwood, P. A. (1959b), *Biochem. J. 73*, 126.
Derechin, S. S., and Johnson, P. (1962), *Nature 194*, 473.
Easley, C. W. (1965), *Biochim. Biophys. Acta 107*, 386.
Edsall, J. T. (1953), *Proteins 1B*, 634.
Ezekiel, E. (1965), *Biochim. Biophys. Acta 107*, 511.
Ezekiel, E., Lai, L. Y. C., and Kaldor, I. (1963), *Comp. Biochem. Physiol. 10*, 69.
Gordon, W. G., Groves, M. L., and Basch, J. J. (1963), *Biochemistry 2*, 817.
Gordon, A. H., and Louis, L. N. (1963), *Biochem. J. 88*, 409.
Gordon, W. G., Ziegler, J., and Basch, J. J. (1962), *Biochim. Biophys. Acta 60*, 410.
Groves, M. L. (1960), *J. Am. Chem. Soc. 82*, 3345.
Harris, J. I., and Hindley, J. (1965), *J. Mol. Biol. 13*, 894.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Jacobs, S. (1962), *Analyst 87*, 53.
Jamieson, G. A. (1965), *J. Biol. Chem. 240*, 2914.
Jeppsson, J.-O., and Sjöquist, J. (1964), *Proc. 6th Intern. Congr. Biochem. II*, 157.
Johansson, B. G. (1958), *Nature 181*, 996.
Johansson, B. G. (1960), *Acta Chem. Scand. 14*, 510.
Jordal, R. (1963), Undersogelser over Transferrin Stofskiltet, Copenhagen, Illemann.
Jordan, S. M., Kaldor, I., and Morgan, E. H. (1967), *Nature 215*, 76.
Kingsley, G. R. (1942), *J. Lab. Clin. Med. 27*, 840.
Koechlin, B. A. (1952), *J. Am. Chem. Soc. 74*, 2649.
Laurell, C.-B. (1953), *Acta. Chem. Scand. 7*, 1407.
Laurell, C.-B., Laurell, S., and Skoog, N. (1956), *Clin. Chem. 2*, 99.
Laurell, C.-B., and Morgan, E. H. (1965), *Biochim. Biophys. Acta 100*, 128.
Michl, H. (1959), *Chromatography 1*, 11.
Montreuil, J., and Mullet, S. (1960), *Compt. Rend. 250*, 1736.
Montreuil, J., Spik, G., Monsigny, M., Deschamps, J., Biserte, G., and Dautrevaux, M. (1965), *Experientia 21*, 254.
Morgan, E. H. (1964), *J. Physiol. 171*, 26.
Morgan, E. H., and Carter, G. (1960), *Australian Ann. Med. 9*, 209.
Morgan, E. H., and Finch, C. A. (1966), *Proc. Soc. Exptl. Biol. Med. 122*, 92.
Muller-Eberhard, H. J. (1960), *Scan. J. Clin. Lab. Invest. 12*, 33.
Oncley, J. L., Scatchard, G., and Brown, H. (1947), *J. Phys. Colloid Chem. 51*, 184.
Parker, W. C., and Bearn, A. G. (1962), *J. Exptl. Med. 115*, 83.
Schafer, K. H. (1951), *Monatsschr. Kinderheilk. 99*, 69.
Scheidegger, J. J. (1955), *Intern. Arch. Allergy Appl. Immunol. 7*, 103.
Schultze, H. E., Schmidtberger, R., and Haupt, H. (1958), *Biochem. Z. 329*, 490.
Smith, H. W. (1965), *J. Pathol. Bacteriol. 90*, 495.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spik, G., and Montreuil, J. (1966), *Compt. Rend. Soc. Biol. 160*, 94.
Surgenor, D. M., Koechlin, B. A., and Strong, L. E. (1949), *J. Clin. Invest. 28*, 73.
Williams, J. (1962), *Biochem. J. 83*, 355.