I wish to express my gratitude to Dr F. Sanger, F.R.S., for his interest and encouragement in this work and to Professor F. G. Young, F.R.S., for generously making laboratory facilities available to me. I am indebted to Professor J. R. Marrack for advice on immunological techniques and for a gift of rabbit anti-ovalbumin serum. Dr D. G. Gilmour kindly supplied me with blood and eggs from birds belonging to the School of Agriculture, Cambridge University. Mr B. Boon carried out the ultra-centrifuge experiments and Mr B. R. Slater the Tiselius electrophoresis experiments. I am grateful to the Medical Research Council for financial support.

REFERENCES

- Abdel-Samie, Y. M., Broda, E. & Kellner, G. (1960). *Biochem. J.* **75**, 209.
- Bürgi, W. & Schmid, K. (1961). *J. biol. Chem.* **236**, 1066.
- Deutsch, H. F. & Goodloe, M. B. (1945). *J. biol. Chem.* **161**, 1.
- Dixon, M. (1953). *Biochem. J.* **54**, 457.
- Glass, L. E. (1959). *J. exp. Zool.* **141**, 257.
- Gorini, I. & Lanzavecchia, G. (1955). *R.C. Ist. lombardo, B*, **88**, 951.
- Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.
- Horesji, J. & Smetana, R. (1956). *Acta med. scand.* **155**, 65.
- Jepson, J. B. & Smith, I. (1953). *Nature, Lond.*, **172**, 1100.
- Jukes, T. H. (1933). *J. biol. Chem.* **103**, 425.
- Jukes, T. H. & Kay, H. D. (1932a). *J. exp. Med.* **56**, 469.
- Jukes, T. H. & Kay, H. D. (1932b). *J. Nutr.* **5**, 81.
- Kaminski, M. & Durieux, J. (1956). *Exp. Cell Res.* **10**, 590.
- Knight, P. F. & Schechtman, A. M. (1954). *J. exp. Zool.* **127**, 271.
- Lineweaver, H., Morris, H. J., Kline, L. & Bean, R. S. (1948). *Arch. Biochem.* **16**, 443.
- McCully, K. A., Maw, W. A. & Common, R. H. (1959). *Canad. J. Biochem. Physiol.* **37**, 1457.
- Mandeles, S. (1960). *J. Chromat.* **3**, 256.
- Marshall, M. E. & Deutsch, H. F. (1951). *J. biol. Chem.* **189**, 1.
- Martin, W. G. & Cook, W. H. (1958). *Canad. J. Biochem. Physiol.* **36**, 153.
- Martin, W. G., Vandegaer, J. E. & Cook, W. H. (1957). *Canad. J. Biochem. Physiol.* **35**, 241.
- Nace, G. W. (1953). *J. exp. Zool.* **122**, 423.
- Onoe, T. (1936). *J. Biochem., Tokyo*, **24**, 1.
- Ouchterlony, Ö. (1949). *Acta path. microbiol. scand.* **26**, 507.
- Patterson, R. (1961). *Fed. Proc.* **20**, 379.
- Plimmer, R. H. A. (1908). *J. chem. Soc.* **93**, 1500.
- Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.
- Schechtman, A. M. (1947). *J. exp. Zool.* **105**, 329.
- Schechtman, A. M. (1956). In *International Review of Cytology*, vol. v, p. 303. Ed. by Bourne, G. H. & Danielli, J. F. New York: Academic Press Inc.
- Scheidegger, J. J. (1955). *Int. Arch. Allergy*, **7**, 103.
- Schjeld, O. A. & Urist, M. R. (1959). *Exp. Cell Res.* **17**, 84.
- Schmid, K. & Bürgi, W. (1961). *Biochim. biophys. Acta*, **47**, 440.
- Schönenberger, M., Kellner, H., Südhoff, H. & Haupt, H. (1958). *Hoppe-Seyl. Z.* **309**, 145.
- Schultze, H. E., Schmidtberger, R. & Haupt, H. (1958). *Biochem. Z.* **329**, 490.
- Shepard, C. C. & Hottle, G. A. (1949). *J. biol. Chem.* **179**, 349.
- Smithies, O. (1955). *Biochem. J.* **61**, 629.
- Sørensen, S. P. L. & Høyrup, H. (1917). *C.R. Lab. Carlsberg*, **12**, 164.
- Svennerholm, L. (1957). *Biochim. biophys. Acta*, **24**, 604.
- Telfer, W. H. (1961). *J. biophys. biochem. Cytol.* **9**, 747.
- Williams, J. (1962). *Biochem. J.* **83**, 355.

Biochem. J. (1962) **83**, 355

A Comparison of Conalbumin and Transferrin in the Domestic Fowl

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(Received 25 September 1961)

It is well known that transferrin, the iron-binding β -globulin of serum, is very similar to conalbumin, the iron-binding protein of avian-egg white. One molecule of either protein readily combines with two atoms of iron and two molecules of carbon dioxide to give a pink complex with an absorption maximum at 460–470 $m\mu$ (Schade, Reinhart & Levy, 1949; Warner & Weber, 1951). In both cases the iron-protein complexes are more resistant to thermal denaturation and to proteolytic degradation than are the iron-free proteins (Azari & Feeney, 1958). Conalbumin and transferrin are also similar in their molecular weights.

Bain & Deutsch (1948) obtained a value of 85 000 for conalbumin by sedimentation and diffusion studies, and iron-binding studies by Warner & Weber (1951) gave a value of 76 600. Laurell & Ingelman (1947) obtained a molecular weight of 88 000 for pig transferrin by sedimentation and diffusion studies, and 90 000 was obtained for human transferrin by Koechlin (1952) with iron-binding and light-scattering methods and by Oncley, Scatchard & Brown (1947) by osmotic and sedimentation measurements.

The iron-binding protein of fowl serum has also been found to be similar to conalbumin. Hektoen

& Cole (1928) observed an immunological relationship between conalbumin and that serum fraction which is soluble in 64% saturated ammonium sulphate and in which most of the transferrin is present. Bruynoghe (1934) confirmed this finding. Later, Marshall & Deutsch (1951) isolated from cock serum a protein which was immunologically identical with conalbumin although its isoelectric point (5.7) was lower than that of conalbumin (6.1). It combined with iron (2 atoms of iron/mol. of protein) and sedimentation and diffusion measurements gave a molecular weight of 72 000–85 000. The sedimentation coefficient ($S_{20,w}^0$ 5.3) was similar to that obtained by Bain & Deutsch (1948) for conalbumin ($S_{20,w}^0$ 5.4). The immunological identity of conalbumin and fowl transferrin was confirmed by Kaminski & Durieux (1956) by immunoelectrophoresis and agar diffusion. They observed that the electrophoretic mobility of the serum protein in agar gel was slightly greater than that of conalbumin.

As reported by Williams (1962), immunoelectrophoretic patterns of livetin showed a precipitin line in the position ascribed by Kaminski & Durieux (1956) to conalbumin or transferrin. The experiments described in the present paper were intended to identify this protein.

An attempt was also made to study the relation between conalbumin and transferrin. In this work the conalbumin from the egg white of some hens whose sera contain modified transferrins was examined. A. L. Ogden, J. R. Morton, D. G. Gilmour & E. M. McDermid (in preparation) examined the serum-transferrin patterns in a large number of hens by means of starch-gel electrophoresis. In some hens the transferrin bands migrated faster at pH 8.6 than the normal transferrin bands, but in others the pattern appeared to be composed of a mixture of the normal slow transferrins with the modified fast transferrins. From the breeding data the former hens were considered to be homozygous for a mutation at the transferrin locus (Tf_a/Tf_a) and the latter to be the heterozygotes (Tf_a/Tf_b). The wild type is homozygous for the allele Tf_b .

EXPERIMENTAL

Materials. Diethylaminoethylcellulose was obtained as Whatman DE50 powder. Carboxymethylcellulose was prepared by the method of Ellis & Simpson (1956). Neuraminidase from *Vibrio cholerae* was a gift from Dr Barry and Dr Waterson; in some experiments a commercial preparation from Behringwerke, Marburg-Lahn, Germany, was used. Radioactive iron (^{59}Fe) was obtained from The Radiochemical Centre, Amersham, Bucks., as $FeCl_3$ in 0.1N-HCl (specific activity 73.9 mc/m-mole). [^{14}C]Arginine (specific activity 25 μ C/mg.) and [^{14}C]lysine (specific activity 52 μ C/mg.) were also obtained from The Radiochemical

Centre. Eggs from hens belonging to Thornber Bros. Ltd. were kindly made available by Mr E. M. McDermid and Dr D. G. Gilmour.

Radioactive labelling with ^{59}Fe . The iron-binding proteins in serum and egg white were located by starch-gel electrophoresis. ^{59}Fe (1 μ C) was added to 0.1 ml. of serum or to 0.1 ml. of egg white before electrophoresis, which was carried out as described by Williams (1962). Then the gels were sliced, wrapped in thin polythene and placed with the cut surfaces in contact with X-ray film. Radioautographs were exposed for 2 or 3 days.

Labelling of egg-white proteins. Small pieces of oviduct from laying hens were incubated with radioactive amino acids by the method of Hendler (1956). Incubation was carried out with shaking for 10–15 hr. at 37°. The sample was then homogenized and centrifuged. The supernatant liquid was streaked on Whatman 3MM paper and subjected to electrophoresis in barbitone buffer, pH 8.6 for 48 hr. After drying, the papers were radioautographed. In these experiments labelling was performed with [^{14}C]lysine or [^{14}C]arginine.

A mince supernatant labelled with [^{35}S]cysteine (Williams & Dawson, 1952) was dialysed against cysteine to remove adsorbed amino acid and then mixed with 0.5 ml. of unlabelled egg white. It was fractionated on a small column of carboxymethylcellulose according to Rhodes, Azari & Feeney (1958) with stepwise elution with solutions of 0.1M-ammonium acetate at pH 4.2, 5.0 and 7.0. The final elution was with 0.2M- Na_2CO_3 . Fractions (2 ml.) were collected and the extinctions at 280 m μ were measured. A sample (0.1 ml.) was taken from each tube and dried on a polythene planchet for measurement of radioactivity.

Preparation of transferrin from livetin. Whole livetin was dissolved in water to make a 2% solution. Solid $(NH_4)_2SO_4$ was added to give 50% saturation (Dixon, 1953). The solution was filtered and more $(NH_4)_2SO_4$ added to give 80% saturation. The resulting precipitate was collected by centrifuging, and dialysed first against water and then against 0.1M-ammonium acetate, pH 5.0. The solution was then passed through a column (2.5 cm. $\times$ 8.0 cm.) of carboxymethylcellulose previously equilibrated with the same buffer. Both α - and β -livetin passed through the column but transferrin was retained as a pink-coloured zone. After washing the column well with 0.1M-ammonium acetate buffer, pH 5.0, the transferrin was eluted with 0.1M-ammonium acetate, pH 7.0. The eluate was dialysed against 0.02M-glycine, pH 6.5, and passed through a column (2.5 cm. $\times$ 5.0 cm.) of diethylaminoethylcellulose which had been equilibrated with 0.02M-glycine, pH 6.5. Transferrin was retained by the cellulose and was eluted with a gradient to 0.02M-glycine–0.02M- KH_2PO_4 –0.02M- K_2HPO_4 ; a mixing vessel containing 150 ml. of 0.02M-glycine was used.

The transferrin peak was dialysed against water and freeze-dried. The carboxymethylcellulose and diethylaminoethylcellulose procedures were based on the schemes described by Rhodes *et al.* (1958) and by Mandeles (1960) respectively for the fractionation of egg-white proteins. Conalbumin was completely adsorbed by carboxymethylcellulose at pH 5.5, whereas transferrin was not adsorbed above pH 5.0.

Preparation of serum transferrin. The procedure described above for the isolation of transferrin from livetin, i.e. precipitation between 50% and 80% saturation with $(NH_4)_2SO_4$

and adsorption on carboxymethylcellulose and diethylaminoethylcellulose, was also used for serum. The purity of the preparations was examined by starch-gel electrophoresis and it was necessary to repeat the chromatography on diethylaminoethylcellulose. The final product was a pink material. In a few experiments serum transferrin was prepared by the rivanol method of Boettcher, Kistler & Nitschman (1958).

Preparation of conalbumin. A combination of the methods of Warner & Weber (1951) and of Rhodes *et al.* (1958) was used. Saturated $(\text{NH}_4)_2\text{SO}_4$ (1 vol.) was mixed with egg white and the mixture was filtered. The filtrate was acidified to pH 4.6 with 0.5N- H_2SO_4 and the precipitated ovalbumin removed by filtration. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the filtrate (8 g./100 ml.) and the conalbumin precipitate collected by centrifuging. The precipitate was dissolved in water and dialysed free of $(\text{NH}_4)_2\text{SO}_4$. The solution (adjusted to pH 6.0 with NH_3) was chilled in ice and 0.67 vol. of 50% (v/v) ethanol in 0.02M-NaCl added. The conalbumin precipitate was dissolved in water and dialysed overnight against water. An excess of Fe^{3+} and HCO_3^- ions were added [as $\text{Fe}(\text{NO}_3)_3$ and NaHCO_3] to form the iron-conalbumin complex. This was dialysed for 2 days against water adjusted to pH 9.0 with NH_3 , filtered and the filtrate dialysed against 0.1M-ammonium acetate, pH 5.5. Conalbumin was then purified by adsorption on carboxymethylcellulose and diethylaminoethylcellulose as described for transferrin. It was finally dialysed against water and freeze-dried.

Amino acid compositions. The amino acid analyses of conalbumin and serum transferrin were carried out in duplicate by the method of Spackman, Stein & Moore (1958). Protein samples were hydrolysed with constant-boiling HCl in sealed evacuated tubes at 105° for 24 hr. The hydrolysates were evaporated to dryness and 1 mg. portions analysed on the Spinco Amino Acid Analyser by Dr R. P. Ambler. Tryptophan was estimated by the spectrophotometric method of Bencze & Schmid (1957).

N-Terminal residues. The N-terminal amino acids of conalbumin and transferrin were determined qualitatively by the fluorodinitrobenzene method (Fraenkel-Conrat, Harris & Levy, 1952).

Starch-gel electrophoresis. This was carried out in the manner described by Williams (1962).

Comparison of peptide patterns. This was carried out as described by Williams (1962), except that the digestions were carried out for 4 hr.

RESULTS

Starch-gel electrophoresis. Starch-gel-electrophoresis patterns of egg white and of laying-hen serum are shown in Fig. 1. Radioautography of samples which had been labelled with ^{59}Fe showed that two bands in the serum (transferrin) and two bands in the egg white (conalbumin) represented iron-binding proteins.

Fig. 2 shows the patterns given by the isolated proteins. Three main bands with different mobilities were present. In conalbumin the slowest band was the most intense but a less intense band was present in the middle position. In some cases a

trace of the fastest band was also visible. Serum transferrin showed three bands which lined up with the conalbumin bands although the relative intensities were reversed: the fastest band was the most intense, the second band less intense and the slowest band was present in only small amounts. Small amounts of a protein which preceded the main transferrin band were also present. The pink protein isolated from livetin gave the same pattern as serum transferrin. In paper electrophoresis at pH 8.6 transferrin migrated a little faster than conalbumin but both proteins migrated as single bands.

Fig. 3 shows schematically the relative positions of the transferrin bands as observed in starch-gel electrophoreses of sera from hens of the three transferrin types Tf_a/Tf_a , Tf_a/Tf_b and Tf_b/Tf_b , as described by A. L. Ogden, J. R. Morton, D. G. Gilmour & E. M. McDermid (in preparation). Egg-white patterns from the three types showed that the transferrin polymorphism was accompanied by a similar polymorphism of conalbumin (Fig. 4). In the homozygous mutants (Tf_a/Tf_a) the main conalbumin band ran faster than the main conalbumin band in the wild type (Tf_b/Tf_b). A minor band was present ahead of the main conalbumin band in both cases. In the heterozygotes (Tf_a/Tf_b) conalbumin was present as two main bands of roughly equal intensities representing the mutant and wild-type conalbumins.

Immunological analysis. In agar diffusion transferrin from serum or livetin and conalbumin reacted with rabbit anti-hen serum to give single precipitin lines which joined one another smoothly. Immunoelectrophoresis of serum transferrin and conalbumin

Fig. 1. Starch-gel electrophoresis of egg white and laying-hen serum. The serum runs show the presence of two pre-albumin bands. Electrophoresis at pH 8.6 was carried out for 4 hr.

with rabbit anti-cock serum gave in each case a single precipitin arc. The anodic mobility of the transferrin was slightly greater than that of the conalbumin (Fig. 5).

Amino acid compositions. The preparations of serum transferrin and conalbumin used for amino acid analysis and comparison of peptide patterns gave single asymmetric peaks in the ultracentrifuge indicating some heterogeneity. Both preparations gave the same sedimentation coefficient (S 1% of 4.5s).

The amino acid compositions of serum transferrin and conalbumin (Table 1) are very similar although the sums of the differences between the columns show that there is more difference between

the conalbumin and transferrin analyses than between the duplicate analyses. In view of the small amount of heterogeneity indicated by the ultracentrifugal patterns, however, this result appears to be consistent with the identity of composition of the two proteins.

Peptide patterns. Tryptic and chymotryptic digests of oxidized samples of transferrin from serum and livetin and of conalbumin were examined by one- and two-dimensional peptide patterns (Figs. 6 and 7). The three samples gave the same patterns.

Some idea of the specificity of the identification of these proteins by means of the peptide patterns

Fig. 2. Starch-gel electrophoresis of transferrin from serum, of pink protein from livetin and of conalbumin. Electrophoresis at pH 8.6 for 4 hr. The three main bands referred to in the text are indicated by 1, 2 and 3.

Fig. 3. Scheme showing the relative mobilities of the transferrin bands in starch-gel electrophoresis of sera of the three transferrin types. a/a, Pattern from the homozygous mutant (Tf_a/Tf_a); a/b, pattern from the heterozygous type (Tf_a/Tf_b); b/b, pattern from the homozygous wild type (Tf_b/Tf_b).

Fig. 4. Starch-gel electrophoresis of egg whites diluted 1:1 with tris-citrate buffer. Egg-white patterns from hens of transferrin types a/a, a/b and b/b (see Fig. 3) are shown. The main conalbumin bands are marked.

is provided by the following experiment (Fig. 8): oxidized samples of rat serum albumin, hen serum albumin, conalbumin and hen ovalbumin were digested overnight with trypsin and the digests subjected to electrophoresis at pH 3.5. The peptides were stained with ninhydrin. Taking account of the positions and relative intensities of the

ninhydrin-positive bands, duplicate digests of each protein gave reproducible patterns by means of which the four proteins were readily distinguished from one another.

N-Terminal residues. In the ether extracts of hydrolysed DNP-conalbumin and DNP-transferrin the only DNP-amino acid detected was DNP-alanine in both cases. Fraenkel-Conrat & Porter (1952) have already reported the *N*-terminal residue of conalbumin to be alanine.

Carbohydrate analyses. Table 2 shows that both conalbumin and transferrin contain carbohydrate. Transferrin contains more hexose than conalbumin does. A little sialic acid is present in transferrin but none was found in either the wild type or mutant conalbumins. The content of hexosamine is the same in both proteins.

Effect of neuraminidase on transferrin. Transferrin (10 mg.) from livetin dissolved in 1 ml. of 0.01M-calcium chloride, pH 5, was treated with 1500 units of neuraminidase at 37°. Samples were removed after different times and stored frozen. After 20 hr. a further 3000 units of enzyme were added to complete the reaction. Starch-gel electrophoresis of the samples (Fig. 9) showed stepwise reduction in the mobilities of the bands. The fastest band disappeared rapidly. The second band at first increased before diminishing and the third band, which was present in only low concentration in the untreated material, steadily increased in intensity until it was the main component present with a trace of the second band in front of it. The position of the slow band corresponded exactly with that of the main band of a conalbumin marker. This result was also obtained with transferrin isolated from serum. A control in which the enzyme was replaced by water showed no change in starch-gel pattern over the same period of time. Thus the enzyme appears to have converted the first transferrin band into the second band and this in turn into the third band. In another experiment transferrin was treated with neuraminidase, as described above, and the solution subsequently deproteinized by the addition of 1 vol. of 10% (w/v) trichloroacetic acid. Analysis of the precipitate and the supernatant solution showed that the sialic acid had been completely released by the neuraminidase.

Neuraminidase caused no change in the electrophoretic pattern of conalbumin. After treatment with the enzyme the mutant and wild-type conalbumins maintained their electrophoretic difference and the minor bands moving ahead of the main conalbumin bands were still present.

Incorporation of amino acids into conalbumin. A radioautograph of a paper electrophoresis of oviduct proteins labelled with [¹⁴C]arginine is shown in Fig. 10. A control strip stained with bromophenol blue showed that the radioactive

Fig. 5. Immunoelectrophoresis of conalbumin and transferrin preparations (each at 0.1%) with anti-cock serum. Cock serum in the centre position (diluted 1:8) shows a well-marked prealbumin arc.

Table 1. *Amino acid compositions of transferrin and conalbumin*

Results are expressed as g. of anhydro amino acid residue/100 g. of protein. Sums of differences:

$$\begin{aligned} \text{Con}_1 - \text{Con}_2 &= 2.42; & \text{Tf}_1 - \text{Tf}_2 &= 2.96; \\ \text{Con}_1 - \text{Tf}_1 &= 4.80; & \text{Con}_1 - \text{Tf}_2 &= 4.48; \\ \text{Con}_2 - \text{Tf}_1 &= 4.66; & \text{Con}_2 - \text{Tf}_2 &= 4.73. \end{aligned}$$

	Conalbumin		Transferrin	
	Con ₁	Con ₂	Tf ₁	Tf ₂
Lys	10.49	10.38	9.98	10.31
His	2.32	2.36	2.00	2.13
Arg	6.71	6.73	6.53	6.89
Asp	11.15	10.97	10.78	10.73
Thr	4.62	4.54	4.52	4.40
Ser	4.82	4.80	4.79	4.71
Glu	11.93	11.72	12.81	12.56
Pro	3.70	4.11	4.12	3.87
Gly	4.36	4.32	—*	—*
Ala	4.89	4.83	4.91	4.86
CySH	2.76	3.27	3.07	2.61
Val	5.76	5.99	5.61	6.19
Met	1.96	1.81	1.94	1.89
Ileu	3.64	3.58	3.45	3.38
Leu	7.20	7.06	7.50	7.54
Tyr	4.39	4.33	4.20	4.22
Phe	4.95	4.92	4.61	4.55
Try	4.35	4.28	4.82	4.85

* The glycine values for transferrin were very high, due probably to insufficient dialysis in the removal of the glycine buffer used in the chromatography on diethylaminoethylcellulose. The glycine values were, therefore, omitted and the remaining values were calculated on the assumption that the amount of glycine in transferrin is the same as in conalbumin.

Fig. 6. Two-dimensional peptide patterns of tryptic hydrolysates of serum transferrin (*A*), pink protein from livetin (*B*) and conalbumin (*C*). The shaded areas represent histidine-containing peptides. Electrophoresis was carried out at pH 6.5 (2000 v for 105 min.). Chromatography was carried out in butan-1-ol-acetic acid-water (3:1:1, by vol.).

Fig. 7. One-dimensional peptide patterns of tryptic and chymotryptic digests of transferrin from livetin (a), of conalbumin (b) and of transferrin from serum (c). A, Chymotryptic digests subjected to electrophoresis at pH 6.5 (2000 v for 105 min.); B, chymotryptic digests subjected to electrophoresis at pH 3.5 (2000 v for 2.5 hr.); C, tryptic digests at pH 6.5 (2000 v for 105 min.). Areas labelled *t* represent tyrosine-containing peptides; areas marked *h* contain histidine. The numbers indicate corresponding peptides.

zones corresponded to protein bands, and the conalbumin band was identified by comparison with a conalbumin marker. When the soluble proteins from a [³⁵S]cysteine-labelled oviduct mince were adsorbed on carboxymethylcellulose the elution pattern shown in Fig. 11 was obtained. Four protein peaks were eluted from the column and each was accompanied by a peak of radioactivity. Peak C was concentrated by freeze-drying and in starch-gel electrophoresis it appeared to consist of conalbumin alone.

DISCUSSION

The starch-gel-electrophoresis pattern shows that the pink protein isolated from livetin is transferrin. This is in agreement with results reported in the preceding paper (Williams, 1962) which show that the protein constituents of livetin correspond to serum proteins.

Schultze (1958) has shown that human transferrin is a glycoprotein and the present analyses show the same to be true for fowl transferrin. Sørensen (1934) reported that conalbumin contained 2.8% of carbohydrate but Bain & Deutsch (1948) found that their preparation of conalbumin did not give the Molisch reaction. The results given here suggest that conalbumin is a glycoprotein containing 2.2% of carbohydrate, although the electrophoretic and ultracentrifugal patterns show that the preparation is not homogeneous.

Fig. 8. One-dimensional electrophoresis of tryptic digests of rat serum albumin (A), hen serum albumin (B), conalbumin (C) and ovalbumin (D). Each digestion was performed in duplicate. Electrophoresis was carried out at pH 3.5, 2000 v for 2½ hr.

The results obtained from amino acid analysis, immunology, and peptide patterns indicate that the protein parts of conalbumin and transferrin are very similar and probably identical. This close relationship is supported by the comparisons of

conalbumins and transferrins from hens bearing a mutant transferrin gene with the corresponding proteins from wild-type hens. The amino acid sequences of transferrin and conalbumin are probably controlled by the same genetic locus.

Transferrin and conalbumin differ in their carbohydrate contents and the result of treating transferrin with neuraminidase suggests that the differences in the electrophoretic mobilities and isoelectric points are due to the sialic acid present in the carbohydrate prosthetic group of transferrin.

Barber & Sheeler (1961) found only one iron-binding protein band in hen serum by starch-gel electrophoresis although in the present work transferrin gave three bands. The conditions of electrophoresis were not identical, however, since Barber & Sheeler (1961) used phosphate buffer at pH 7.0. The heterogeneity of the transferrin in starch-gel electrophoresis is probably due to the presence of different amounts of sialic acid, since neuraminidase converted the various bands into a band with the mobility of conalbumin which lacks sialic acid. The analytical value of 0.35 % of sialic acid for a transferrin preparation is not in satisfactory agreement with this idea, however, since if a molecular weight of 90 000 is assumed only

1 residue of sialic acid/mol. of protein is allowed for.

Since Warren & Spearing (1960) and Ada & Lind (1961) have recently shown that neuraminidase activity is present in some tissues of higher animals it seemed possible that conalbumin might arise in the hen's oviduct by the removal of sialic acid from the carbohydrate prosthetic group of transferrin. The experiments on the labelling of oviduct proteins with radioactive amino acids suggest, however, that the oviduct can incorporate amino acids into conalbumin as well as into other egg-white proteins, and Mandeles & Ducay (1961) also observed incorporation of [^{14}C]glycine into conalbumin by an oviduct mince. It is not known where transferrin is synthesized although it may be in the liver. In the rat a liver homogenate incorporated radioactive valine into the transferrin fraction although a spleen homogenate did not do so (A. H. Gordon, personal communication). The present results suggest that the transferrin gene operates in at least two different organs in the fowl, namely the oviduct and the liver.

The effects of neuraminidase on fowl transferrin are different from those which have been obtained with mammalian transferrins. Several authors have observed a fall in the electrophoretic mobility of human transferrin after treatment with neuraminidase, and starch-gel electrophoresis has shown splitting of the transferrin band into a variable number of bands: Blumberg & Warren (1961) obtained two, Poulik (1959) three and Parker & Bearn (1961) five bands. In homozygous cattle of types AA, DD or EE, starch-gel electrophoresis has shown that each transferrin is represented by three bands (Ashton, 1958). In some unpublished experiments A. L. Ogden (personal communication) has

Table 2. *Carbohydrate compositions of conalbumin and transferrin*

Results are expressed as percentages.

	Con- albumin	Trans- ferrin
Hexose	0.8	2.8
Hexosamine	1.4	1.4
Sialic acid	0	0.35

Fig. 9. Starch-gel electrophoresis of transferrin treated with neuraminidase for different times. The pattern from a conalbumin marker (C) is also shown. Electrophoresis at pH 8.6 was carried out for 4 hr.

Fig. 10. Radioautograph of a paper electrophoresis of oviduct proteins labelled with [^{14}C]arginine. The conalbumin band is marked.

found that treatment of these sera with neuraminidase reduces the mobilities of all the transferrin bands without affecting their number or relative intensities.

SUMMARY

1. A pink protein, identified by starch-gel electrophoresis as transferrin, has been isolated from the livetin fraction of hen's-egg yolk.

2. Conalbumin and transferrin have been compared by means of starch-gel electrophoresis, peptide patterns, immunoelectrophoresis, amino acid compositions and carbohydrate analyses.

3. The results suggest that conalbumin and transferrin are glycoproteins which differ only in

Fig. 11. Elution of oviduct proteins labelled with [^{35}S]cysteine from carboxymethylcellulose. Stepwise elution with solutions of increasing pH is shown by the arrows. The solid line shows E_{280} ; the broken line shows the radioactivity.

their carbohydrate prosthetic groups. They appear to be identical in their main protein parts.

I am very grateful to Dr F. Sanger, F.R.S., for his advice and encouragement and to Dr R. P. Ambler for performing the amino acid analyses. I am also grateful to Mr A. L. Ogden, Dr D. G. Gilmour, Mr J. R. Morton and Mr E. M. McDermid for allowing me to use some of their data and to the Medical Research Council for financial support.

REFERENCES

- Ada, G. L. & Lind, P. E. (1961). *Nature, Lond.*, **190**, 1169.
- Ashton, G. C. (1958). *Nature, Lond.*, **182**, 370.
- Azari, P. R. & Feeney, R. E. (1958). *J. biol. Chem.* **232**, 293.
- Bain, J. A. & Deutsch, H. F. (1948). *J. biol. Chem.* **172**, 547.
- Barber, A. A. & Sheeler, P. (1961). *Comp. Biochem. Physiol.* **2**, 233.
- Bencze, W. L. & Schmid, K. (1957). *Analyt. Chem.* **29**, 1193.
- Blumberg, B. S. & Warren, L. (1961). *Biochim. biophys. Acta*, **50**, 90.
- Boettcher, E. W., Kistler, P. & Nitschman, H. (1958). *Nature, Lond.*, **181**, 490.
- Bruynoghe, G. (1934). *C.R. Soc. Biol., Paris*, **115**, 441.
- Dixon, M. (1953). *Biochem. J.* **54**, 457.
- Ellis, S. & Simpson, M. E. (1956). *J. biol. Chem.* **220**, 939.
- Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1952). *Meth. biochem. Anal.* **2**, 359.
- Fraenkel-Conrat, H. & Porter, R. R. (1952). *Biochim. biophys. Acta*, **9**, 557.
- Hektoen, L. & Cole, A. G. (1928). *J. infect. Dis.* **42**, 1.
- Hendler, R. W. (1956). *J. biol. Chem.* **223**, 831.
- Kaminski, M. & Durieux, J. (1956). *Exp. Cell Res.* **10**, 590.
- Koechlin, B. A. (1952). *J. Amer. chem. Soc.* **74**, 2649.
- Laurell, C. B. & Ingleman, B. (1947). *Acta chem. scand.* **1**, 770.

- Mandeles, S. (1960). *J. Chromat.* **3**, 256.
 Mandeles, S. & Ducay, E. D. (1961). *Fed. Proc.* **20**, 389.
 Marshall, M. E. & Deutsch, H. F. (1951). *J. biol. Chem.* **189**, 1.
 Oncley, J. L., Scatchard, G. & Brown, A. (1947). *J. phys. chem.* **51**, 184.
 Parker, W. C. & Bearn, A. G. (1961). *Science*, **133**, 1014.
 Poulik, M. (1959). *J. Immunol.* **82**, 502.
 Rhodes, M. B., Azari, P. R. & Feeney, R. E. (1958). *J. biol. Chem.* **230**, 399.
 Schade, A. L., Reinhart, A. W. & Levy, H. (1949). *Arch. Biochem.* **20**, 170.
 Schultze, H. E. (1958). *Dtsch. med. Wschr.* **83**, 1742.
 Sørensen, M. (1934). *Biochem. Z.* **269**, 271.
 Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
 Warner, R. C. & Weber, I. (1951). *J. biol. Chem.* **191**, 173.
 Warren, L. & Spearing, C. W. (1960). *Biochem. biophys. Res. Commun.* **3**, 489.
 Williams, J. (1962). *Biochem. J.* **83**, 346.
 Williams, R. B. & Dawson, R. M. C. (1952). *Biochem. J.* **52**, 314.

Biochem. J. (1962) **83**, 364

Intracellular Localization of the Skeletal-Muscle Relaxing Factor

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(Received 12 October 1961)

The relaxing factor, originally described by Marsh (1951) in the crude extract of blended skeletal muscle, has been shown to be associated with minute particles which can only be centrifuged out of suspension at high centrifugal forces (Kumagai, Ebashi & Takeda, 1955; Portzehl, 1957). Portzehl (1957) observed that, after centrifuging at 19000g for 1 hr., the supernatant solution still contained relaxing factor as assayed by inhibition of myofibril adenosine triphosphatase activity, whereas centrifuging at 35000g for over 1 hr. brought down all the active granules. On the basis of these characteristics she postulated that the granular fraction responsible for relaxing activity consisted of microsomes.

With passage of time along this postulate has become an assumption (Lorand, Mulnar & Moos, 1957; Briggs & Fuchs, 1960). However, support for this assumption was presented by Nagai, Makinose & Hasselbach (1960), who showed that the granules which centrifuged down at 25000g in 1 hr. were vesicular and had an average diameter of about 1000 Å. The electron micrograph of these muscle granules is quite similar in appearance to the microsome pellet of liver (Palade & Siekevitz, 1956).

The present work was undertaken to determine, by means of enzyme markers, the nature of the skeletal-muscle granules which possess the relaxing activity.

METHODS

Myofibrils. Rabbit leg and back muscles were chilled, ground, and blended for 2 min. in a Waring Blendor with 5 vol. of 0.1M-KCl-0.01M-KHCO₃ soln. The homogenate

was centrifuged at 2000g for 10 min., the supernatant discarded, and the sedimented fibrils blended for 30 sec. with 5 vol. of 0.1M-KCl. After sedimenting the fibrils, the blending for 30 sec. with 5 vol. of 0.1M-KCl was repeated eight times in all; this broke up the fibrils into small segments and washed them free of relaxing factor. All steps were carried out at 0-4°.

After the final blending, the myofibrils were filtered through four thicknesses of gauze to remove lumps of fibrous tissue, and centrifuged for 10 min. To the precipitate was added cold glycerol (1 vol.) and sufficient 3.0M-KCl to give a final KCl concn. of 0.1M. The pH was adjusted to 7.0 by the addition of 0.1N-KOH. This mixture was then blended for 15 sec. to yield a uniform suspension and was stored at -10°. On the day of an experiment a portion of the glycerol suspension was centrifuged, and the myofibril precipitate washed once with 0.1M-KCl and recentrifuged. It was then suspended in 0.1M-KCl at pH 7.0 to give a protein concentration of about 5 mg./ml. Essentially no change in adenosine triphosphatase activity occurred in the 7-8 months that the myofibril preparation was used.

Muscle-granule preparation. Diced muscle (100 g.) from the hind legs of a freshly killed rabbit was chilled, ground and blended at high speed with 2 vol. of a cold 0.1M-KCl-0.02M-KHCO₃-0.005M-potassium oxalate soln. for 1 min. The blended muscle (fraction W.H.) was then centrifuged at 600g for 4 min., the precipitate discarded, and the supernatant (fraction S₁) centrifuged at 3500g for 10 min. The precipitate was gently homogenized by hand with 10-15 ml. of 0.1M-KCl-0.001M-potassium oxalate-0.02M-histidine soln. at pH 7.0 (histidine mixture), recentrifuged for 10 min. at 3500g and the supernatant added to that of the first 3500g centrifuging. The precipitate was taken up in a small volume of the histidine mixture and homogenized by hand to yield an even suspension. This particulate fraction is referred to below as the 'mitochondrial 1' or 'M₁' fraction. The combined supernatants were then centrifuged at 12500g for 20 min. The sediment was washed and suspended as described for the M₁ fraction above, recentrifuged at 12500g for 20 min. and suspended

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