The Presence of Vitamin A in Human Tryptophan-Rich Prealbumin

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(Received 27 June 1966)

Human tryptophan-rich prealbumin has been isolated from serum and its homogeneity evaluated by immunoelectrophoresis and polyacrylamide-gel electrophoresis. The green fluorescence of the protein when exposed to ultraviolet light was shown to be due to the presence of vitamin A. It is suggested that tryptophan-rich prealbumin represents the hitherto unknown specific transport protein for vitamin A.

In connexion with work on the fractionation of uric acid-binding proteins in human serum (Alvsaker, 1966) a study was also made of fluorescent serum proteins. The reason for this is our interest in a novel fluorescent substance isolated from liver (Laland *et al.* 1966). It was observed that one particular protein fraction invariably exhibited green fluorescence. Further studies of this protein fraction showed that the fluorescence was associated with the tryptophan-rich prealbumin and that it was due to attached vitamin A. The present work suggests this protein to be the previously unknown transport protein for vitamin A.

MATERIALS AND METHODS

Sera. The main work was carried out on blood from a healthy male aged 26 years. In addition, blood from five healthy donors was used (three males and two females). Post-absorptive blood was withdrawn by venous puncture and the blood was allowed to clot for 2 hr. at room temperature followed by 4 hr. at 4°. To obtain the serum, the sample was centrifuged for 30 min. at 4° in an MSE refrigerated centrifuge.

Horse immune serum against human serum was purchased from the Central Laboratorium van de Bloedtransfusiedienst, Van Het, Nederlandsche Roode Kruis, Amsterdam W, The Netherlands. Rabbit immune serum against human prealbumin was obtained from Behringwerke A.-G., Marburg-Lahn, Germany. This specific prealbumin antiserum invariably gave, when human serum was analysed immunoelectrophoretically, one precipitation line situated in the prealbumin area.

Tryptophan-rich prealbumin. Material isolated by the method of Schultze, Schöenberger & Schwick (1956) was kindly supplied by Dr Schwick of Behringwerke A.-G. An impure sample of the thyroxine-binding tryptophan-rich prealbumin, containing 10% of albumin, was kindly supplied by Dr J. H. Oppenheimer of Montefiore Hospital and Medical Center, New York, N.Y., U.S.A.

Chromatographic procedures. The sera were subjected to

gel filtration on a Sephadex G-200 column (6 cm. diam. $\times$ 60 cm. high; void volume 600 ml.) in 0.2 M-phosphate buffer, pH 7.35, containing NaCl (1 M).

Fractionation of the components in the third macromolecular fraction (C; Fig. 1) from the gel filtration was achieved by ion-exchange chromatography on a DEAE-Sephadex A-50 (medium grade) column (2 cm. diam $\times$ 30 cm. high). The column was equilibrated with 0.02 M-phosphate buffer, pH 7.35. The material was applied on the column in this buffer and eluted by increasing stepwise the concentration of NaCl.

Electrophoresis. Immunoelectrophoresis was carried out according to the micro-method of Scheidegger (1955). The immunoelectrophoresis equipment of LKB Produktor A.B., Stockholm, Sweden, was used. The supporting medium was 2% (w/v) Difco Special Agar Noble in barbital buffer, pH 8.4–8.6 and 1.0–0.5. Before immunoelectrophoresis, the protein fractions were dialysed against distilled water, freeze-dried and dissolved in 0.9% NaCl.

Paper electrophoresis was carried out on Whatman no. 3MM paper in 0.05 M-acetate buffer, pH 4.0, containing NaCl (0.08 M). Electrophoresis on polyacrylamide gel was carried out according to the methods described by Ornstein (1964) and Davis (1964) at pH 9.4 and 4.3 respectively.

Immunodiffusion. This was carried out according to the micro-technique of Crowle (1958). A 1% (w/v) solution of Difco Special Agar Nobel in 0.9% NaCl was used.

Fluorescence measurements. Most measurements were carried out with a Farrand spectrofluorometer (Farrand Optical Co. Inc., New York, N.Y., U.S.A.). Filters 7-54 and 3-73 were used in the position between the lamp and exciter monochromator and the sample and the analyser monochromator respectively. Approximately the true excitation spectrum of the protein-vitamin A complex was recorded on an automatically corrected fluorescence spectrophotometer (Børresen, 1965). The fluorescence spectrum was recorded on the same instrument and corrected according to the spectral sensitivity distribution of this instrument.

Ultraviolet-absorption measurements. The ultraviolet spectrum of vitamin A was recorded in a Unicam SP.500 spectrophotometer.

Extraction of vitamin A from the protein fraction. The method used was based on a published procedure (Associa-

tion of Vitamin Chemists, 1951). For the liberation of vitamin A, 50mg. of fluorescent protein was mixed with 10ml. of 2% (w/v) KOH in 90% (v/v) ethanol. The mixture was hydrolysed for 30 min. on a water bath at 90° protected from light. The hydrolysate was then extracted three times with light petroleum (b.p. 40–60°) 10ml. each time. The pooled extracts were further extracted with water to remove traces of KOH and then concentrated to dryness and dissolved in 5ml. of the desired solvent.

Purification and identification of vitamin A. A modified Carr–Price test (Carr & Price, 1926) was carried out in chloroform with a 20% (w/v) solution of SbCl_3 .

The anhydrovitamin A test was carried out according to the method of Budowski & Bondi (1957).

Vitamin A was purified on alumina (for chromatography; E. Merck A.-G., Darmstadt, Germany) by the method of Baggesgaard-Rasmussen *et al.* (1956).

RESULTS

Purification of the fluorescent protein. Fractionation of serum on Sephadex G-200 (Fig. 1) gave the well-known elution pattern (Gelotte, Flodin & Killander, 1962). Three fluorescent peaks (A, B and C) were found (Fig. 1). The fluorescent substances in the first two peaks have been studied previously (Laland *et al.* 1966). The material in the third fluorescent peak (C) was further fractionated on a DEAE-Sephadex column. The elution pattern is recorded in Fig. 2. The blue fluorescent fractions I,

II and III all had excitation maxima at 360m μ and fluorescence maxima at 440m μ (Farrand). The fourth green fluorescent fraction (IV) had excitation maximum at 330m μ and fluorescence maximum (uncorrected) at 470m μ (Farrand).

The other five sera (see the Materials and Methods section) were fractionated on Sephadex G-200 and DEAE-Sephadex and gave in each case a green fluorescent peak corresponding to peak IV in Fig. 2. When the fluorescent fraction (IV) was tested immunoelectrophoretically at pH 8.4–8.6 by using antiserum to human serum, five to seven precipitation lines were seen. These were situated mainly in the position of α_1 - and α_2 -globulin (Fig. 3a, 2). When this fraction was tested against anti-(human prealbumin) serum, a precipitation line corresponding to the position of prealbumin was seen (Fig. 3b, 2).

The fluorescent fraction (IV) was dissolved (50mg. of protein/ml.) in 0.05M-sodium acetate buffer, pH 4.0 containing sodium chloride (0.08M) and dialysed against the same buffer for 24 hr. at 4°. Then 0.1ml. of this solution was applied to Whatman no. 3MM paper (4cm. wide $\times$ 40cm. long) and electrophoresis carried out in the above buffer at 4° for 20 hr. at 5v/cm. The green fluorescent band was eluted with water and freeze-dried. The material was dissolved in the above sodium acetate

Fig. 1. Fractionation of serum on Sephadex G-200. A 40ml. sample was subjected to gel filtration on a column (6cm. diam. $\times$ 60cm. high) in 0.2M-phosphate buffer, pH 7.35, containing NaCl (1M). The flow rate was 50ml./hr. and 25ml. fractions were collected. The ultraviolet absorption of the fractions was recorded at 280m μ (histogram). The fluorescence of the fractions was also recorded (●—●) at optimum conditions for excitation and fluorescence.

Fig. 2. Fractionation of the material in peak C (Fig. 1) on DEAE-Sephadex A-50. The pooled fractions (fractions 23-40; Fig. 1) were dialysed overnight against 10l. of distilled water at 4° to remove most of the salts and then subjected to gel filtration on a Sephadex G-25 column (3 cm. diam. $\times$ 30 cm. high) equilibrated with 0.02M-phosphate buffer, pH 7.35. The protein fraction in the 0.02M-phosphate buffer was then adsorbed on the DEAE-Sephadex A-50 column (2 cm. diam. $\times$ 30 cm. high) previously equilibrated with the same buffer. The column was eluted with 0.02M-phosphate buffer containing the following concentrations of NaCl: 0.10M, 0.15M, 0.20M, 0.25M, 0.40M and 1.0M. The flow rate was approx. 15 ml./hr. and 25 ml. fractions were collected. The protein elution pattern (histogram) was recorded at 280 $m\mu$ and the visible fluorescence (●---●) recorded at optimum conditions for excitation and fluorescence.

Fig. 3. Immunoelectrophoresis of the fluorescent fractions. In (a) and (b) antiserum to human serum and the specific prealbumin antiserum respectively were used. In 1 and 2 electrophoretically purified fluorescent protein and material in fluorescent peak IV (Fig. 2) respectively were used.

buffer and dialysed against this buffer for 24 hr. at 4°. The dialysed material was further purified by renewed electrophoresis on paper under the same conditions as above. Under ultraviolet light the strip exhibited a zone of green fluorescence extending cathodically over an area 1-1.6 cm. from the line of application. The strip was stained with Amido Black 10B; the area with the fluorescent zone stained heavily.

The fluorescent zone from unstained strips was eluted with 0.01M-phosphate buffer, pH 7.35, containing sodium chloride (0.5%) and corrected excitation and emission spectra were recorded (Fig. 4): there is an excitation maximum at 330 $m\mu$ and the fluorescence maximum occurs at 495 $m\mu$ (470 $m\mu$, uncorrected). These spectra were identical with those given by the fluorescent material before electrophoresis. Excitation at 280 $m\mu$ (maximum for tryptophan) gave a fluorescent peak with a maximum at 340 $m\mu$ (fluorescence maximum for tryptophan).

Fig. 4. Corrected excitation (A) and fluorescence (B) spectra of the prealbumin fraction (obtained after paper electrophoresis). The excitation spectrum was obtained by examining the fluorescence at $495\text{ m}\mu$. The fluorescence spectrum was recorded after excitation at $330\text{ m}\mu$.

The purity of the fluorescent protein fraction obtained after repeated paper electrophoresis in acetate buffer, pH 4.0, was tested. When it was tested by polyacrylamide-gel electrophoresis at pH 9.4, one band corresponding to the mobility of prealbumin and a barely visible band of albumin were found. The sample of tryptophan-rich prealbumin supplied by Dr Schwick gave a major band with mobility equal to that in our preparation. In addition, two faint bands and a somewhat stronger band, the latter of which corresponded to albumin, were seen. When run at pH 4.3, the same pattern of bands was obtained. In immuno-electrophoresis with our tryptophan-rich prealbumin preparation no precipitation lines developed with the antiserum to human serum (Fig. 3a, 1), but a distinct precipitation line developed with the specific prealbumin antiserum (Fig. 3b, 1). The absence of a precipitation line with the isolated prealbumin when the antiserum to human serum was used is well known and is the result of the weak antigenic properties of prealbumin (Grabar & Burtin, 1964). Double immunodiffusion in agar of our tryptophan-rich prealbumin against the antiserum to prealbumin and comparison with the sample of the thyroxine-binding tryptophan-rich prealbumin and the tryptophan-rich prealbumin, isolated by the method of Schultze *et al.* (1956), gave one line only and patterns of fusion corresponding to reactions of identity. From these experiments it appears that our preparation was tryptophan-rich prealbumin and that it was essentially pure. Thus the protein responsible for the fluorescence was tryptophan-rich prealbumin.

Identification of the fluorescent substance. To identify the substance responsible for the fluorescence in the prealbumin fraction, material from peak

Fig. 5. Ultraviolet spectrum in propan-2-ol of purified vitamin A isolated from material in peak IV (Fig. 2).

IV obtained after elution with 0.25 M-sodium chloride (Fig. 2) was used.

A 12 mg. sample of the protein was saponified (see the Materials and Methods section) and extracted with light petroleum. After evaporation, the residue was dissolved in chloroform. The antimony trichloride product of this solution exhibited maximum absorption at $620\text{ m}\mu$, which is identical with that given by vitamin A-antimony trichloride complex. Part of the chloroform solution was concentrated to dryness and dissolved in benzene. The anhydrovitamin A test of this solution gave a product that showed absorption maxima at 378 and $398\text{ m}\mu$, in good agreement with 377 and $399\text{ m}\mu$ for the derivative from pure vitamin A.

A 50 mg. sample of protein was saponified and extracted with chloroform. The extract was purified on a column of alumina. The fluorescent eluate was dissolved in 5 ml. of propan-2-ol and the absorption spectrum of the solution recorded (Fig. 5). As expected, the maximum absorption was at $325\text{ m}\mu$, and the ratios of the extinctions at 310 and $335\text{ m}\mu$ to that of $325\text{ m}\mu$ were 0.854 and 0.853 respectively, the value for pure vitamin A in both cases being 0.857 (Baggesgaard-Rasmussen *et al.* 1956). Thus the substance responsible for the fluorescence was vitamin A. The ultraviolet spectrum suggests that it is vitamin A alcohol (Kofler & Rubin, 1960).

DISCUSSION

The tryptophan-rich prealbumin isolated in the present work seems to be identical with the prealbumin isolated by the technique of Schultze *et al.* (1956). Further, the present results show that vitamin A is responsible for the fluorescence exhibited by the human tryptophan-rich prealbumin. Thus tryptophan-rich prealbumin seems to be a carrier protein for vitamin A.

Previous reports have shown that vitamin A alcohol is found mainly in its free form in post-absorptive blood, and that the carrier protein is not a lipoprotein but a sedimentable protein (Krinsky, Cornwell & Oncley, 1958). The present result does not contradict these reports. On the other hand, our own results do not support the suggestions that vitamin A is carried by albumin (Hack, 1956), that it migrates with the α_1 -globulin (Garbers, Gillman & Peisach, 1960) or that it is attached to a protein very similar to caeruloplasmin in the α_2 -globulin fraction (Glover & Walker, 1964).

Whether or not vitamin A is covalently linked in the tryptophan-rich prealbumin is not known. No investigation of the stoichiometric relationship between the two components in the complex has been attempted.

The observation that tryptophan-rich prealbumin (Schultze *et al.* 1956) serves as the transport protein for vitamin A alcohol is noteworthy, since it demonstrates a hitherto unknown function of this protein in the human body. Until the present work, the only physiological function attributed to Schultze's tryptophan-rich prealbumin has been the transport of thyroxine as demonstrated by Oppenheimer, Surks, Smith & Squef (1965). They found that each prealbumin molecule has a single thyroxine-binding site and suggested that this protein has a rather specific biological function. However, the present work suggests that vitamin A is also attached to this protein. Thus this protein seems to transport two very different substances.

This observation raises the question whether competition for tryptophan-rich prealbumin exists between thyroxine and vitamin A. It is noteworthy that Moore (1957) in his monograph describes

clinical observations that suggest antagonism between vitamin A and thyroxine.

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