The quenching of flavin fluorescence by electrolytes has been studied by Theorell & Nygaard (1954) and by Rutter (1958). It has been found to be a function both of ionic strength and species. Our data give evidence for an unfavourable influence of Na^+ ion on fluorescence, especially on the dinucleotide. Szent-Györgyi (1957) noticed that the fluorescence of the dye in the liquid transudate from a muscle stained with acridine orange and suspended in potassium chloride or in sodium chloride is quenched by Na^+ ion, and he attributed this fact to an influence of Na^+ ion on water structures.

As seen from Fig. 2 a linear relationship of fluorescence with flavin concentration is found up to values far above those usually found in samples when measured (not more than $1 \mu\text{g./ml.}$). A similar relationship was found by Yagi (1957) for the fluorescence of flavin spots on paper. This fact allows the use of an internal standard for any extract analysed. This is not possible with the Bessey *et al.* (1949) method, where no proportional increase of fluorescence is found at higher flavin concentrations.

When working under uniform conditions standard curves can be drawn with known amounts of flavin. The use of an internal standard is, however, safer and simpler.

SUMMARY

1. The influences of flavin concentration, ionic strength and different ions on the fluorescence of

riboflavin, riboflavin 5'-phosphate, riboflavin 5'-pyrophosphate and flavinadenine dinucleotide were studied.

2. A method has been worked out for the extraction and separate determination of these compounds from animal tissues, and the results are compared with those obtained after proteolytic digestion of the tissue.

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The Transport of Vitamin A in Rat Serum with Special Reference to the Occurrence of Unidentified Metabolites of Vitamin A in the Rat

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Despite the suspected association of vitamin A alcohol with serum proteins, the mechanism of vitamin A transport in serum is still not fully known. Bennhold (1938) was led to believe that both vitamin A and the carotenoids were associated with the globulins, a view which was later

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supported by Pett & le Page (1940) after alcoholic fractionation of serum. According to Dzialszynski, Mystkowski & Stewart (1945), however, vitamin A was said to accompany those proteins of the blood which were precipitated by full or three-quarter saturation with ammonium sulphate. Accordingly it was inferred that vitamin A was most likely attached to albumin.

The interpretation of results obtained from ammonium sulphate fractionation of chicken, cow and pig plasma is complicated by large losses, particularly of vitamin A ester (Ganguly, Krinsky, Mehl & Deuel, 1952). Nevertheless, it appeared from the observations of Ganguly *et al.* (1952) that 40–60% of the vitamin A alcohol in chicken, cow and pig plasma could be recovered from the proteins precipitating at half-saturation, whereas the vitamin A esters seemed to be associated with the proteins precipitated at lower salt concentrations. After fractionation of chicken and rat plasma by dialysis against distilled water, vitamin A ester was found with a protein fraction which precipitated more readily on dialysis than did the protein carrying vitamin A alcohol.

While our work was in progress, Krinsky, Cornwell & Oncley (1956) reported that during flotation of the lipoproteins of human plasma in the ultracentrifuge the vitamin A alcohol-protein complex sedimented together with the other proteins. From their studies on the transport of vitamin A and of the carotenoids in human plasma Krinsky, Cornwell & Oncley (1958) arrived at the view that since albumin, isolated by two different experimental procedures, did not contain significant amounts of vitamin A alcohol, some protein other than albumin was the carrier of the vitamin A in the blood.

The available evidence would suggest therefore that vitamin A is made soluble in the blood by association with a protein.

With the aid of improved techniques now available for the fractionation of serum proteins by preparative electrophoresis and with the aid of [^{14}C]vitamin A, it is hoped in this investigation to identify the vitamin A-carrying serum protein (Garbers, Gillman & Peisach, 1958). Thereafter we shall draw attention to the presence of uncharacterized products of vitamin A in the rat's serum and their possible importance in metabolism.

MATERIALS AND METHODS

[^{14}C]Vitamin A. This was prepared from [^{14}C]vitamin A acid by reduction with lithium aluminium hydride after esterification with diazomethane (Garbers, 1956). The [^{14}C]vitamin A alcohol (radioactivity: 1.07 mc/m-mole) was dissolved in purified hexane, stabilized with DL- α -tocopherol (5%) and stored at -20° under N_2 until used for the preparation of doses.

Preparation of doses. Peanut oil was extracted with ethanol, residual ethanol in the oil was removed *in vacuo* and the oil then distilled in a 5 in. rotating disk molecular still (Distillation Products Inc., Rochester, N.Y., U.S.A.). The first third of the distillate was discarded and the residue distilled at 200–220° at 0.03 mm. Hg. α -Tocopherol was added to the oil to the extent of 0.1 mg./100 g. [^{14}C]Vitamin A was dissolved in this oil so that

each dose (0.2 ml. of oil) contained 100 i.u. of labelled vitamin A.

Treatment of experimental animals. Male albino rats of the Wistar strain were used in all experiments. Vitamin A-deficient animals were produced by feeding the following diet to weanling animals: casein, 15%; dextrinized corn starch (corn starch cooked in boiling water, dried at a low temperature and ground to a fine powder), 71%; brewer's yeast, 10%; Steenbock's salt mixture no. 40 (NaCl , 9.49%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10%; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 14.59%; K_2HPO_4 , 28.28%; CaHPO_4 , 28.29%; calcium lactate pentahydrate, 6.26%; iron citrate hexahydrate, 2.44%; KI, 0.65%), 4%.

(i) Expts. 1–3 and 5. After 5–6 weeks on the vitamin A-free diet the rats started to lose weight, and fat (5–10%) containing vitamin A (Gillman, Norton, Rivett & Sutton, 1956) was included in the diet. The amount of fat consumed by the rats per day contained the equivalent of 2 i.u. of vitamin A/day. With this amount the rats failed to grow (or grew only slowly), but continued to live. When the amount of vitamin A supplemented was decreased, the rats gradually lost weight and died. Before use the animals were fed with the vitamin A-free diet until signs of deficiency developed, whereupon they received five successive daily doses of 100 i.u. of [^{14}C]vitamin A in 0.2 ml. of refined peanut oil by stomach tube. Groups of 5, 15, 6 and 18 rats were used for Expts. 1–3 and 5 respectively.

(ii) Expt. 4. A group of 10 male albino rats (average wt. 34 g.) was weaned on a vitamin A-free diet as above. When these rats started to lose weight (average wt. 157 g.), the diet was supplemented twice weekly with 175 i.u. of [^{14}C]vitamin A in peanut oil for 6 weeks (average weight at the end of this period 221 g.). The vitamin A administered allowed marginal storage in the liver and a normal blood-vitamin A concentration (Lewis, Bodansky, Falk & McGuire, 1942).

Collection of blood. The rats were anaesthetized with ether 2–43 days after administration of the last dose and blood was collected from the dorsal aorta. The blood was allowed to clot for 2 hr. and the serum was collected and pooled. Under these experimental conditions the serum contained only vitamin A alcohol with little or no vitamin A esters. The serum for electrophoresis fractionation was dialysed at 2° for 12 hr. against the buffer used for the electrophoresis separation. The vitamin A concentrations of the serum did not decrease during the dialysis.

Vitamin A analyses. Serum proteins were denatured by addition of an equal volume of absolute ethanol. The serum-ethanol mixture was extracted once with an equal volume of light petroleum (b.p. below 40°) followed by two further extractions with half the volume of light petroleum (b.p. below 40°). The extracts were combined and evaporated under N_2 in an Evelyn tube, and the vitamin A in the residue was estimated by the Carr-Price colour reaction with the Evelyn spectrophotometer.

Liver samples were ground with Na_2SO_4 and extracted with ether according to the method of Ames, Risley & Harris (1954). The turbidity which sometimes developed after the addition of the Carr-Price reagent was eliminated by filtering a measured portion of the ether extract through alumina and eluting the vitamin A with hexane-ethanol (92:8, v/v) (Thompson, Ganguly & Kon, 1949).

If the vitamin A concentration in serum or tissue was to be calculated from radioactive assay, the vitamin was

extracted similarly, the solvent evaporated and the residue was subjected to combustion. The $^{14}\text{CO}_2$ formed was isolated as $\text{Ba}^{14}\text{CO}_3$. If required, samples of serum or tissue were also made directly to produce $^{14}\text{CO}_2$ by combustion, and $\text{Ba}^{14}\text{CO}_3$ was isolated.

Vitamin A-breakdown products in the urine. Repeated samples of urine were obtained from vitamin A-deficient rats by making a fistula in the bladder. The neck of the bladder immediately below the entrance of the ureters was isolated by blunt dissection and a small incision made in the ventral wall. A polyethylene tube (no. 1) was passed through the incision into the bladder and tied in position with non-absorbable thread. The cannula was exteriorized and led into a glass saddle affixed to the back of the rat (van Zyl, 1957). After 1–2 days the urine was clear and its flow regular. Four days after the operation the rats were fed by stomach tube 500 i.u. of $[2\text{-}^{14}\text{C}]\text{vitamin A}$ in 0.2 ml. of peanut oil. Through the use of a special metabolism cage (C. F. Garbers & C. Gilbert, unpublished work) urine samples were collected from the saddle and blood samples from the tail at fixed intervals. The blood and urine samples were subjected to combustion separately and the $\text{Ba}^{14}\text{CO}_3$ was isolated and analysed.

Water and the vitamin A-free diet were supplied without restriction to the experimental animals throughout the experiment.

Combustion of samples and assay of radioactivity. Combustion of samples was according to the method of Evans & Huston (1952), the oxidizing mixture of van Slyke, Plazin & Weisiger (1951) being used. The CO_2 formed during the combustion was isolated as BaCO_3 and weighed. The CO_2 was generated from the BaCO_3 and absorbed in N-NaOH . From this solution the carbonate was precipitated as BaCO_3 on 20 cm^2 planchets (approx. 3 mg. of $\text{BaCO}_3/\text{cm}^2$; Peisach, 1959) and counted with 34% efficiency in a proportional-counter converter (Nuclear Measurements Corp., type P.C.C.-10) screened in 3 in. of steel; gas: Tracerlab proportional gas. The results are expressed as counts/min. and are corrected for self-absorption.

Concentration of lipoproteins from rat serum. This was effected by preparative ultracentrifuging of rat serum at solution densities of 1.063 and 1.20 g./ml. in the Spinco model E analytical ultracentrifuge according to the method of de Lalla & Gofman (1954). The lipoprotein fractions were changed by combustion directly to CO_2 for radioactive assay. The sedimented proteins were dialysed against borate buffer, pH 8.6, to remove most of the KBr before combustion.

Fractionation of rat-serum proteins. The method as described by Porath (1956), in which electrophoresis is carried out on columns from which the proteins are subsequently eluted with buffer, was used. The supporting medium was partially acetylated cellulose powder (Campbell & Stone, 1956, 1957). Two columns were used: column *a* (2 cm. $\times$ 32 cm.), washed with borate-phosphate buffer (0.0115 M- $\text{Na}_2\text{B}_4\text{O}_7$ -0.0155 M- NaH_2PO_4 , pH 8.6, *I* 0.05), was developed for 18 hr. at 18 mA and column *b* (2.2 cm. $\times$ 39 cm.), washed with veronal buffer (0.1 M-sodium diethylbarbiturate-0.02 M-diethylbarbituric acid, pH 8.6, *I* 0.05), was developed for 40 hr. at 15 mA. The proteins were eluted at a rate of 10–15 ml./hr. Volume of fractions collected: 2.0–2.1 ml. All these manipulations were carried out at 2°. The buffer used was first kept *in vacuo* to remove dissolved gases and then saturated with N_2 .

The protein content of the fractions was determined by measurements of *E* at 280 $\text{m}\mu$ in a Beckman DU or Unicam SP. 600 spectrophotometer. The homogeneity of the fractions was tested by paper electrophoresis or sedimentation experiments or both.

Concentration of protein solutions for sedimentation experiments. If the protein solutions in the borate-phosphate buffer were too dilute for sedimentation experiments, the fractions were concentrated by evaporation in a vacuum desiccator. The proteins dissolved in the veronal buffer were precipitated with ethanol in the presence of Zn^{2+} ions (Cohn *et al.* 1950) as follows: the protein solution eluted from the column with veronal buffer was mixed with an equal volume of precipitating solution (11.502 g. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g. of acetic acid and 400 ml. of 95% ethanol, diluted to 1000 ml. with water) at -5° . In trial experiments it was found that the pH, measured with the aid of a glass electrode (instrument standardized against phosphate buffer), of the resulting protein suspension diluted with 4 ml. of NaCl solution (0.02 M) was approx. 6.0 (Cohn *et al.* 1950). After precipitation the supernatant was kept, the precipitate was freeze-dried and dissolved in disodium ethylenediaminetetra-acetate solution [3.308 g. of disodium ethylenediaminetetra-acetate and 77.6 ml. of 0.1 N-NaOH made to 1000 ml.; pH 7.0 (measured with the aid of a glass electrode), *I* 0.05].

Sedimentation. Sedimentation runs were made in the Spinco model E analytical ultracentrifuge at room temperature and at top speed (59 780 rev./min.). After the sedimentation runs, all the mother liquors and protein solutions of the fraction analysed were subjected to combustion and assayed for radioactivity.

Paper electrophoresis. Whatman no. 3 MM paper was suspended horizontally over a Perspex rack in a labyrinth-type of apparatus (van Kampen & Zondag, 1955). Proteins were stained with bromophenol blue (Hardwicke, 1954) and the glycoproteins with the periodic acid-Schiff stain (Björnesjö, 1955).

RESULTS

Zone electrophoresis of rat-serum proteins

Expt. 1. Electrophoresis fractionation of rat-serum proteins with borate-phosphate buffer (Campbell & Stone, 1956, 1957; Porath, 1956): mobilities of the α_1 -glycoprotein and the macroglobulin. Fig. 1 shows the distribution obtained when rat-serum proteins, which were collected from rats 3 days after administration of the last dose, were fractionated by electrophoresis on a partially acetylated cellulose-powder column. A component migrating faster than the albumin in the electric field can be readily observed. Two consecutive fractions, nos. 7 and 8, were pooled and their homogeneity and nature investigated by paper electrophoresis and by sedimentation runs in the ultracentrifuge. Fig. 2 indicates the sedimentation-boundary diagrams of fractions 7 and 8. Fractions 7–18 were investigated similarly and the results of the analysis (Fig. 1) showed that the albumin peak consisted of at least three components, namely the albumin, the macroglobulin and the third com-

ponent which migrated faster than the albumin under the experimental conditions. This last-named component stained positive for a glycoprotein by the method of Björnesjö (1955) and possessed the sedimentation properties of a globulin. When this component was re-examined by paper electrophoresis in the veronal buffer (pH 8·6, *I* 0·05), it possessed a mobility slightly less than that

Fig. 1. Zone-electrophoresis fractionation on a cellulose-powder column (2 cm. × 32 cm.) of rat-serum proteins (3 ml.) with the borate-phosphate buffer (pH 8·6, *I* 0·05). A current of 18 ma was applied for 17 hr. ●, Total protein; △, albumin; ▲, α₁-glycoprotein; ○, macroglobulin. Histograms indicate radioactivity. The curve for the α₁-glycoprotein was obtained from the experimentally determined points together with the assumption that at any point (from fractions 6 to 16) the sum of the concentrations of the albumin, α₁-glycoprotein and macroglobulin (determined with the aid of sedimentation experiments) equalled the concentration of the total protein (determined spectroscopically).

Fig. 2. Sedimentation-boundary diagrams of the combined fractions 7 and 8 in borate-phosphate buffer. Speed 59 780 rev./min. Exposures A, B, C and D were taken 8, 32, 56 and 80 min. respectively after reaching top speed. a, Macroglobulin; b, glycoprotein; c, albumin.

Table 1. Correlation between the vitamin A concentrations of rat-serum as calculated from Carr-Price measurements and radioactive assay

Values for the vitamin contents of serum are the averages of duplicate analyses unless indicated otherwise.										
		Vitamin A (i.u./100 ml. of serum)								
Expt.	No. of rats	No. of days between feeding of last dose and collection of serum	Radioactive assay on fat-soluble components			Radioactive assay on serum	Percentage of activity not extractable	<i>b/a</i>	Counts/min. on α ₁ -globulin (<i>c</i>)	Counts/min. on globulins (<i>d</i>)
			Carr-Price measurements	(<i>a</i>)	(<i>b</i>)					
1 (Fig. 1)	5	3	111	112	121	7·5	1·08	2247	2247	1
2 (Fig. 3)	15	2	101	—	131	23*	1·30*	1446	1977	1·37
3 (Fig. 4)	6	4	53†	45·6†	75	39·2	1·64	970	1530	1·58
4 (Fig. 6)	7	7	Lost	135	189·8	29	1·41	2440	3640	1·49
4	3	43	74†	66·7	86·2	22·6	—	—	—	—
5	13	2	101	—	134	24·6*	—	—	—	—
5	5	10	73	—	100·1	27·1*	—	—	—	—

* Calculated from radioactive assay and Carr-Price measurements on serum.

† Results from single analysis on serum.

of the albumin and is therefore most likely an α_1 -glycoprotein (Garbers & Joubert, 1958). Analyses of the fractions revealed the radioactivity to be associated with the α_1 -globulin. The radioactivity is not associated with the albumin, since one would then expect the radioactivity to be proportionally distributed over the albumin peak (Fig. 1).

The vitamin A content of the serum in Expt. 1 calculated from Carr-Price measurements and radioactive assay on whole serum and the fat-soluble components from serum agree well (Table 1).

Expt. 2. Electrophoresis fractionation of rat-serum proteins with borate-phosphate buffer: isolation of the lipoproteins. Fig. 3 shows the result when the serum-protein fractions eluted from the column after electrophoresis were analysed for radioactivity. The radioactivity was identified not only on the α_1 -globulin but also on other globulins. From the latter approx. 27% of the total radioactivity was eluted (Table 1). It is possible that some of the radioactivity was carried on the β -globulin (Fig. 3).

The lipoproteins from the same serum were also isolated by flotation in the ultracentrifuge according to the method of de Lalla & Gofman (1954). The radioactivity was associated neither with the low- nor with the high-density lipoproteins, but with the proteins which sediment during flotation of the

lipoproteins in the ultracentrifuge. The results are summarized in Table 2.

The vitamin A content of the serum obtained by radioactive assay exceeded the vitamin A level as determined by Carr-Price colour reaction by 29.7% (Table 1).

Expt. 3. Electrophoresis fractionation of rat-serum proteins with veronal buffer: mobility of the macroglobulin in this buffer. Fig. 4 shows the distribution obtained when rat serum, obtained from the rats 4 days after administration of the last dose, was fractionated by zone electrophoresis in veronal buffer.

In Expt. 1 it was established that when the borate-phosphate buffer is used for the electrophoretic fractionation of rat-serum proteins, two components (macroglobulin and α_1 -glycoprotein) migrated faster than the albumin. The ultracentrifugal analysis of the pooled fractions 15 and 16 of this experiment reveals that when the non-complexing veronal buffer is employed for the

Fig. 3. Zone-electrophoresis fractionation of rat-serum proteins (3 ml.). Conditions were identical with those of Fig. 1. A current of 18 mA was applied for 18 hr. ●, Total protein. Histograms indicate radioactivity.

Fig. 4. Zone-electrophoresis fractionation of rat-serum proteins (3.5 ml.) on a cellulose-powder column (2.2 cm. $\times$ 36 cm.) with veronal buffer (pH 8.6, I 0.05). A current of 15 mA was applied for 36 hr. ●, Total protein. Histograms indicate radioactivity.

Table 2. Isolation of lipoprotein fractions

Description of fractionation	Radioactivity (%) in	
	Lipoprotein fraction	Sedimented proteins
Ultracentrifugal flotation fractionation at solution density 1.063	1.1	98.1
Ultracentrifugal flotation fractionation at solution density 1.20	3.9	94.2

Fig. 5. Sedimentation-boundary diagrams of the combined fractions 15 and 16 in ethylenediaminetetraacetic acid solution. Speed 59 780 rev./min. Exposures A, B, C and D were taken 8, 32, 56 and 80 min. respectively after reaching top speed. a, Macroglobulin; b, albumin.

electrophoresis of rat serum, only the macroglobulin is found to migrate faster than the albumin (Fig. 5).

Analysis of the protein fractions revealed that approx. 63.4% of the radioactivity recovered in these fractions was associated with the α_1 -globulin and the remainder with other globulins.

Here again the vitamin A concentrations differed when determined by Carr-Price colour reaction and by radioactive assay on the whole serum. It was found that 39.2% of the radioactivity was not extractable from serum by the conventional methods employed during the Carr-Price estimation (Table 1).

Expt. 4. Electrophoresis fractionation of rat-serum proteins with veronal buffer: mobility of the α_1 -glycoprotein in this buffer. The vitamin A administered to the rats in Expts. 1-3 and 5 was not sufficient to allow storage. The association of vitamin A with the serum proteins in rats having reserves of vitamin A in the liver was investigated. Under the experimental conditions described, the rat livers contained an average of 31.2 i.u. of vitamin A/g. of liver (range 21-44 i.u./g.) 1 week after administration of the last dose. The analysis of the serum is reported in Table 1.

A procedure similar to that described in Expt. 3 was followed for the fractionation of a sample of pooled serum obtained from a group of seven rats 1 week after administration of the last dose. When the protein fractions eluted from the column after electrophoresis were analysed, 67% of the radioactivity recovered was associated with the α_1 -globulin and the remaining 33% with the other globulins (Fig. 6).

The position of the α_1 -glycoprotein was determined by spotting on paper portions of the

fractions, obtained after electrophoresis and staining for glycoproteins, followed by elution and subsequent quantitative estimation of the dye as recommended by Björnesjö (1955). As is expected from an α_1 -globulin component, the mobility of the glycoprotein is smaller than that of the albumin (Fig. 6). The presence of globulin components could also be demonstrated by paper-electrophoresis techniques in fractions 14-20 of the albumin peak. In the veronal buffer the macroglobulin still migrates faster than the albumin (Expt. 3), whereas in the borate-phosphate buffer both the α_1 -glycoprotein and the macroglobulin migrate faster than the albumin during electrophoresis (Expt. 1).

It has been observed before that the mobilities of certain components in sera change in buffers containing borate (Consden & Powell, 1955; Goldwasser & Mathews, 1955; Aronsson & Grönwall, 1957; Cooper, 1958). This increased mobility of the α_1 -glycoprotein is most likely due to the action of the borate ion with the glycol groupings, leading to increased charges on the glycoproteins. As the position of the peak of radioactivity on the α_1 -globulin is unaltered when either the borate-phosphate buffer or the veronal buffer is employed, the radioactivity is probably also not associated with the α_1 -glycoprotein.

Influence of time on the amount of radioactivity not extractable from serum

The results obtained in Expts. 1-3, where serum samples were collected 3, 2 and 4 days respectively after administration of the last dose of labelled vitamin A, indicated that the radioactivity could not always be completely extracted by the conventional methods used for the extraction of vitamin A from serum. A similar investigation (Expt. 5) verified these results, and a difference of 24.6 and 27.1% still existed between the vitamin A concentration of the serum determined by Carr-Price colour reaction and that calculated from radioactive assay 2 and 10 days after administration of the last dose (Table 1).

Similarly, in Expt. 4, where the experimental animals had reserves of labelled vitamin A in the liver, a discrepancy was found between the radioactivity in whole serum and the radioactivity in the fat-soluble components extracted from serum 1 week after administration of the last dose. To assess whether this discrepancy is persistent in serum, three rats from this group were maintained on a vitamin A-free diet for a period of 43 days after administration of the last dose. During this period the rats continued to grow (average increase in weight: 22 g.), and at the end of this period 29.2% more radioactivity was found in whole serum than in the fat-soluble components extracted from the serum (Table 1).

Fig. 6. Zone-electrophoresis fractionation of rat-serum proteins (4 ml.) on a cellulose-powder column (2.2 cm. $\times$ 36 cm.) with the veronal buffer (pH 8.6, 1.0-0.5). A current of 15 ma was applied for 40 hr. ○, Total protein; △, glycoprotein. Histograms indicate radioactivity.

Comparison of the concentrations of vitamin A in rat serum as calculated from radioactive assay and from Carr-Price measurements on serum

In the paragraph 'Treatment of experimental animals' conditions are described that enabled us to create a physiological condition which allowed the animal to survive without having any vitamin A in the blood or tissues as judged by the Carr-Price colour reaction. Administration of 500 i.u. of labelled vitamin A to these animals resulted in the appearance of vitamin A in the blood. This vitamin A was all labelled, since the vitamin A content of the fat-soluble components extracted from the serum was of the same order whether determined by radioactive assay or Carr-Price colour reaction. However, on occasions not all the radioactivity was confined to the fat-soluble extracts. In other words, part of the radioactivity derived from the labelled vitamin A was present as a component (or components) not extractable from serum by conventional methods. Furthermore, this second labelled component(s) could not be separated from the serum by dialysis.

The normal occurrence of this second labelled component(s) in the serum was suspected from the results of Expts. 4 and 5. Despite the varying conditions under which these experiments were conducted, this second labelled component(s) was still present in quantities varying from 23 to 29 % of the total radioactivity in serum (Table 1).

The large amount of radioactivity (39.2 %) which could not be extracted from the serum in Expt. 3 was found in rats greatly depleted of vitamin A before the labelled vitamin A was fed. In this

connexion it should be mentioned that during the period of depletion and before radioactive vitamin A was fed one rat developed panophthalmia.

If the results presented in Table 1 are considered with the data obtained from the electrophoresis fractionation of the serum proteins, it is evident that: (i) one peak of radioactivity on the α_1 -globulin is to be found when the vitamin A concentrations of the serum calculated from the radioactive assay and from the Carr-Price estimation correlate well (Fig. 1); (ii) a discrepancy between the vitamin A concentration calculated from total radioactivity and from the Carr-Price measurements on whole serum was found with two peaks of radioactivity, namely one on the α_1 -globulin and the second on other globulins.

No proof can be offered that part or all of the radioactivity eluted from the column was indeed present as vitamin A. However, the correlations presented in Table 1 more than suggest that the fat-soluble components extracted from the ethanol-denatured serum contain vitamin A alcohol as the only radioactive component on the α_1 -globulin. The radioactivity not extractable from the ethanol-denatured serum is associated mostly with the α_2 -globulin.

Breakdown products of vitamin A in the urine

Labelled water-soluble products (11.7 % of the dose) derived from [2- 14 C]vitamin A are excreted in the urine of rats within 24 hr. after the intraperitoneal injection of 9200 i.u. of [2- 14 C]vitamin A to depleted rats (Wolf, Kahn & Johnson, 1957).

Under our experimental conditions the excretion of radioactivity in the urine reached a maximum during the period 4-10 hr. after the oral administration of 500 i.u. of [2- 14 C]vitamin A (Fig. 7). In the two experiments conducted, the radioactivity excreted in the urine during the 48 hr. after oral administration was 6.8 and 11.5 % of the administered dose. The bulk of this radioactivity, 69.4 and 82.4 % respectively, was excreted during the first 24 hr. period.

The radioactivity in the blood reached a maximum after approx. 4 hr., and at the end of 48 hr. the livers of the animals contained radioactivity corresponding to 20.6 and 19.8 % respectively of the administered dose.

DISCUSSION

Our investigations in the rat disclose that vitamin A alcohol is transported by a globulin component of the serum protein. In this respect the transportation of vitamin A in the rat is similar to that described for man by Krinsky *et al.* (1958). In the rat the globulin component could be still further characterized. The vitamin A alcohol appears to

Fig. 7. Appearance of radioactivity in the blood and urine of vitamin A-deficient rats after administration of 500 i.u. of [2- 14 C]vitamin A. Rat 1: ●, radioactivity in whole-blood samples; ○, radioactivity in urine samples. Rat 2: ▲, radioactivity in whole-blood samples; △, radioactivity in urine samples.

accompany the α_1 -globulin but probably not the α_1 -glycoprotein.

The finding that vitamin A alcohol is not associated with the lipoproteins clearly dissociates the transporting mechanism of vitamin A alcohol from that of the vitamin A esters, which are transported in rat serum, as in man, by the low-density lipoproteins (Garbers, 1958).

The amount of vitamin A in rat serum, as determined by Carr-Price colour reaction, cannot always account for all the radioactivity in the serum after the oral administration of labelled vitamin A to depleted rats. Another component(s) derived from vitamin A therefore is present in the blood. Unfortunately, in this investigation no evidence can be presented as yet whether there is one or more such component. The results to date indicate that in the serum of rats with little or no reserves of vitamin A the concentration of this second component(s) is equivalent (in terms of radioactivity) to approx. 20–30 i.u. of vitamin A/100 ml. of serum. The transport of the second component(s) derived from vitamin A seems to be different from that of vitamin A alcohol.

The evidence for and against the presence in serum of an intermediary product of vitamin A not detectable by the methods usually employed for the chemical estimation of vitamin A has been reviewed (Moore, 1957). Experiments suggest that on feeding vitamin A to depleted rats the vitamin A absorbed is first transformed into an intermediary product which in turn is utilized for promoting growth and for preventing xerophthalmia (Moore, 1957). Once this intermediary product reaches a particular concentration, vitamin A alcohol may be expected in the blood and ultimately in the liver and kidneys.

A situation can be visualized where rats are depleted of their stores of vitamin A and eventually also of the vitamin A in the blood. In such circumstances the intermediary product nevertheless may still be present in the blood, thus accounting for survival of vitamin A-deficient rats despite the failure to demonstrate vitamin A in the blood by conventional methods. On feeding labelled vitamin A to these rats, the labelled vitamin A again appears in the blood and depots. However, in short-term experiments the intermediary product may still not be labelled. In such circumstances there may be adequate amounts of the intermediate, i.e. the more immediately utilizable form of vitamin A. Accordingly the vitamin A administered to the depleted rat is not required for metabolism and therefore the vitamin A concentration rises in the blood and also later in the depots. On the other hand, depending on the state of depletion of the animal and the amount of labelled vitamin A fed, a situation could arise where there is

so little of the vitamin A intermediate present in the blood that the feeding of labelled vitamin A would lead rapidly to the formation and appearance in the blood of the labelled vitamin A intermediate at the same time as the labelled vitamin A alcohol accumulates in the blood. The finding that this second labelled component(s) occurs in varying quantity in rat serum does not necessarily exclude it (them) from being a normal product(s) of vitamin A metabolism.

le Gallic (1947) provided suggestive experimental evidence for the presence in the blood of albino rats of a component having vitamin A bioactivity, but which was not detectable by the Carr-Price colour reaction. Since le Gallic's experiments and the investigations of others of the 'lard factor', the so-called 'hidden forms' of vitamin A have received renewed attention. It has now been shown that herring eggs also possess more bioactivity than can be accounted for by the Carr-Price colour reaction (Fisher *et al.* 1956). Subsequently, Plack, Thompson & Kon (1958) and Plack, Kon & Thompson (1959) identified vitamin A aldehyde as a constituent of herring eggs. Evidence which might indicate the presence of retinene in the rat's liver was also presented (Scharpenseel & Wolf, 1958).

In our experiments, this second labelled component(s) in the rat serum may be merely a breakdown product(s) of vitamin A and not an active intermediate product(s) of vitamin A. It is known that compounds similar in structure to vitamin A are absorbed, transported in the blood and stored in the liver (Ames, Swanson & Harris, 1955).

The rat normally does not excrete any vitamin A in the urine. Furthermore, the labelled products in the urine after feeding [2- 14 C]vitamin A are water-soluble (Wolf *et al.* 1957) and may be either the active intermediate(s) or merely a breakdown product(s) thereof or of vitamin A. If the labelled products in the urine are breakdown products of vitamin A, they are most likely derived from the circulating blood. However, there is a possibility that the labelled products are elaborated and excreted by the kidneys or, less likely, by the urinary passages. However, if the component(s) in the urine is the same as that in the serum, it follows from the results obtained that the kidneys have the ability to concentrate this component(s), especially as the average excretion of radioactivity/unit volume in the urine is greater than in the blood, where the radioactivity is due to [2- 14 C]-vitamin A and the other component(s). As the synthetic vitamin A was labelled at C₂, the second labelled component(s) in the serum is to be expected to be relatively large or to be covalently linked to the proteins, otherwise it (they) should separate during dialysis.

Whether this second labelled component(s) in rat serum belongs to the hidden forms of vitamin A or whether it (they) is merely a decomposition product is now being investigated in this Laboratory.

SUMMARY

1. With the aid of [2-¹⁴C]vitamin A it was established that vitamin A alcohol is transported in rat serum in association with the α_1 -globulin. The vitamin A alcohol is not associated with the lipoproteins.

2. When a borate-phosphate buffer is employed for the electrophoretic fractionation of rat-serum proteins, the mobility of the α_1 -glycoprotein is greater than that of the albumin.

3. By employing veronal buffer and borate-phosphate buffer for the electrophoretic fractionation of rat-serum proteins, it could be concluded that vitamin A alcohol is probably also not associated with the α_1 -glycoprotein.

4. A labelled component(s) derived from [2-¹⁴C]-vitamin A and associated with the α_2 -globulin can occur in serum and most likely is a normal constituent of serum, occurring in a concentration equivalent to 20–30 i.u. of vitamin A. It has not been possible as yet to establish whether these derivatives of vitamin A are active intermediates or degradation products.

5. On giving [2-¹⁴C]vitamin A orally to vitamin A-depleted rats, 7–12% of the radioactivity is excreted in the urine within 48 hr. after administration.

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