figures in the second decimal place are only approximate but have been retained in order to allow the best possible estimate of the O_2 :Fe ratio.) The haemoglobin equivalent of the artificial standard based on oxygen capacity and iron determinations is given for each blood in the remaining columns.

Our results indicate that there is no difference in the O_2 :Fe ratio between men and women. The numbers examined were not large, but the average of the individual O_2 :Fe ratios was identical in each sex. The mean value corresponded to 393 ml. O_2 /g. Fe, the values ranging from 388 to 398 ml. in men and 386 to 399 ml. in women. The coefficients of variation were 0.8% in men and 0.9% in women. (The plasma Fe content is too small to affect the above results and has been neglected.)

The values for the artificial standard agree well with those derived from ten male bloods in our previous paper, the present value based on oxygen capacity being 15.78 as against 15.60 g. of haemoglobin/100 ml., and the present value from iron being 16.11 as against 16.01 found previously. The average values for the standard, based on determinations on all thirty bloods are 15.7 g. from oxygen and 16.1 g. of haemoglobin/100 ml. from Fe. We have found that there is no difference between the sexes with regard to the ratio between iron and colour and oxygen and colour, so that the same haemoglobin equivalent may be used for the standard in haemoglobin determinations carried out on the blood from persons of either sex.

To estimate the mean blood haemoglobin level of the medical students who assisted us by acting as donors in our work, the values for the ten men

who were tested in the work already published (Gibson & Harrison, 1945) were combined with those for the ten men tested on this occasion. Assuming an equivalent of 14.75 g. Hb/100 ml. for the British Standards Institution Haldane standard (Macfarlane & O'Brien, 1944; King, Gilchrist & Matheson, 1944), and using our oxygen capacity figures for the bloods, the mean Haldane value for the twenty men students was 107 (s.d. (standard deviation) 7). The blood samples were taken in 1945, and the value is almost the same as that found for fifty-two men students in 1943 in the haemoglobin survey carried out in England by the Medical Research Council Committee on Haemoglobin Surveys (1945). The average in the English survey was 106.4 (s.d. 6.4) on the British Standards Institute Haldane scale. The corresponding value for our small series of ten women medical students was 101 (s.d. 6) as compared with 92.8 (s.d. 6.8) from fifty-nine women students in the English survey.

SUMMARY

1. The ratio between oxygen capacity and Fe content is the same in the blood of men and women. The specific oxygen capacity found was 393 ml. O_2 /g. Fe (standard error of mean 0.8 ml.) compared with the stoichiometric value of 401 ml.

2. The artificial standard may be used with the same values for blood from either sex.

3. Values for the artificial standard agree closely with those previously published, the mean values from thirty bloods being 15.7 g. of haemoglobin/100 ml. of blood from oxygen capacity, and 16.1 g. from Fe.

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Micro-method for the Determination of Vitamin A in Liver Biopsies in Man and Larger Animals

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Since the liver contains over 95% of the vitamin A stored in the organism several problems concerning the metabolism of vitamin A have been elucidated by means of analyses of liver tissue—mainly in small animals, large numbers of which can be used in experiments (With, 1940, 1942). For economical

reasons experiments in which animals are killed have been few on larger animals, such as ox, horse, sheep, and swine, and many problems still remain. In man, the storage of vitamin A in the liver has not been seriously studied, although investigations might be possible by means of operation biopsies

combined with analyses of necropsy material. If it were possible to determine the vitamin A content of the minute biopsies taken by means of liver puncture (Iversen & Roholm, 1939), such investigations could be made and, moreover, the vitamin A content of the liver followed at short intervals.

Since the weight of the liver can be regarded as constant during short periods, without committing errors of importance the concentration of vitamin A in the biopsy can be reckoned to be proportional to the vitamin A content of the liver. It is important that the biopsy be taken under standard conditions—for instance, fasting in the morning—in order to exclude errors due to variation in the water content and other possible rhythmical diurnal variations in the composition of the liver.

In this paper is described a method for the estimation of vitamin A in biopsies of liver and a comparison of the results which it yielded with those obtained on autopsy material.

METHODS

By reading the Carr-Price reaction in special small cuvettes ('Kleinkyvetten') it is possible to obtain sufficiently thick layers to get measurable extinctions with the minute amounts of liver tissue of the micro-biopsies. According to Roholm, Krarup & Iversen (1942) such biopsies obtained by the method of Iversen & Roholm (1939) are cylinders of 2×15 mm., weighing about 50 mg. As the vitamin A content of human liver normally is 25–1000 i.u./g. (With, 1940, 1941*b*), it is now possible to calculate the Carr-Price extinction of the vitamin A in such liver biopsies.

If a liver contains 25 i.u./g. a biopsy of 50 mg. contains 1.25 i.u., which after extraction are dissolved in 2.5 ml. of chloroform. If the Carr-Price reaction is performed with the least possible dilution, i.e. equal amounts of solution and reagent, the 1.25 i.u. are dissolved in 5 ml. of reaction mixture, and this is enough for two readings in 5 cm. Kleinkyvetten. If the liver reserves are greater, the unsaponifiable fraction of the biopsy is dissolved in 5–10 ml. of chloroform, and greater amounts of reagent may be used, e.g. 0.2 ml. solution and 0.5 ml. of reagent, 0.1 + 1.0 ml., or 0.02 + 1.0 ml.

Since a surplus of reagent is required smaller amounts of reagent than the volume of the test solution give too low extinctions, and it is only in the case of weak solutions that equal volumes are sufficient to give quantitative reaction. Measurements on solutions containing 0.5–2 i.u./ml. of vitamin A showed with an equal volume of reagent the same extinctions as with greater volumes, provided that the extinctions were calculated for the same volume.

From measurements of the Carr-Price reaction and ultraviolet spectral absorption of liver extracts, the extinction value of 1.0 in the Carr-Price reaction—measured with the filter S61 of the Pulfrich photometer in a 1 cm. thick layer—corresponds to a vitamin A content of c. 600 i.u./100 ml. of the reaction mixture (Engel, Granström, Lindgren &

Norlander, 1942; Jensen & With, 1939; With, 1940). Thus 1.25 i.u. dissolved in 5 ml. must give an extinction of about 0.21 in a 5 cm. thick layer according to Beer's law.

It is possible to read the extinction of the Carr-Price reaction of the vitamin A in aspiration biopsies of the liver even if its vitamin A concentration lies at the lower limit of the normal. In this case, however, the analytical error is considerable, since only two readings of the Carr-Price reaction are possible, but if the liver reserve is greater the analysis can be carried out with an error of $\pm 5\%$ (With, 1940), and this will most often be the case in man and larger animals.

Procedure

If necessary the biopsy specimen is cleansed of adhering blood with a bit of filter paper. A small piece of the specimen may be taken for histological examination to ascertain that it really is liver tissue. Then the specimen is weighed and, if the analysis cannot be made immediately, is placed in a small vessel filled with N_2 or CO_2 .

The saponification and extraction, etc., are carried out as previously described (With, 1940). The biopsy specimen is heated carefully with 3 ml. of 5% ethanolic KOH in a little flask which is shaken for a few minutes to produce solution of the tissue. The heating must be as short as possible and 'roasting' has to be avoided. Then the contents of the flask as well as the distilled water with which it is washed are placed in a 50 ml. separatory funnel and extracted three times with 10 ml. of ether free from peroxides. The combined ethereal extracts are washed in the usual way and dried by suction through a firmly compressed layer (c. 1 cm. thick) of anhydrous Na_2SO_4 upon a sintered glass filter (e.g. 3 G3); the sulphate is washed twice with 5 ml. of dry ether. The dry ether is then quickly evaporated in a small flask in the presence of N_2 or CO_2 or *in vacuo*; the heating must be mild and short. The residue is dissolved in 1 ml. of freshly distilled chloroform (A.R.), transferred to a 10 ml. measuring cylinder with glass stopper; the flask is washed three times with 0.5 ml. of chloroform and these washings added to the cylinder and the volume adjusted to 2.5 ml. The chloroform solution is used for the determination of vitamin A by the Carr-Price reaction; it may stand several hours in a dark ice-box before the measurement, without loss of vitamin A (Fuchs & Soos, 1943).

Colorimetric measurements are made with the Pulfrich photometer equipped with Kleinkyvetten and filter S61 (With, 1940). At first an orientating experiment is carried out on 0.1 ml. of solution and 0.1 ml. of reagent in a test tube. If this gives a pale-blue colour the reaction is carried out in a 5 cm. Kleinkyvette with 1.25 ml. solution and 1.25 ml. reagent, but if the colour is more intense, 0.1 ml. solution and 1.0 ml. reagent are used—or 0.02 + 1.0 ml., etc.—and 2, 1, or 0.5 cm. Kleinkyvetten according to the intensity of the colour. The 5, 2, 1 and 0.5 cm. Kleinkyvetten require respectively 2.5, 1.0, 0.5 and 0.25 ml. of fluid. If the orientating reaction shows turbidity, one or two drops of acetic anhydride are placed in the bottom of the cuvette before the reading. The control cuvette is filled with chloroform.

The amounts of reagent and solution and the layer of thickness are selected so that the extinction lies between

0.3 and 0.8. The average of four readings of separate mixtures of solution and reagent gives the final result. The first reading—within 5 sec.—of each mixture is used. To obtain rapid and complete mixing of test solution and reagent in the narrow Kleinkyvetten it is essential to blow gently through the reagent pipette, which has to reach close to the bottom of the cuvette.

Measurement of the carotenoids is unnecessary as they are present only in negligible amounts in human liver (With, 1940).

The calculation is then made with the following formula:

$$E_{1\text{ cm}}^{1\%} (\text{S61}) = \frac{1000 \times E \times (S + R)}{L \times S \times B \times 40},$$

in which *B* (in mg.) is the weight of the biopsy, *S* (in ml.) the volume of the solution, *R* (in ml.) that of the reagent, *L* (in cm.) the thickness of layer, and *E* the extinction reading. *E*_{1 cm.}^{1%} is the extinction of the unsaponifiable residue of 1 g. of liver tissue dissolved in 100 ml. of solution-reagent mixture and measured in a 1 cm. thick layer. The formula is valid only if the unsaponifiable residue of the biopsy is dissolved in 2.5 ml. of chloroform. The concentration in i.u./g. of liver is calculated by multiplying *E*_{1 cm.}^{1%} by 600, mentioned above (With, 1940, 1941*a*).

The photometer used was calibrated by measurements of the standard vitamin A preparation ‘Vogan’ (Merck) (cf. Willstaedt & With, 1938) and further controlled by comparison of the Carr-Price readings with spectrographic measurements of the 328 mμ extinction in a Hilger quartz spectrograph with echelon cells (Jensen & With, 1939; With, 1940).

RESULTS

Comparison between the micro-method and a macro-method

As the vitamin A concentration of the liver varies (Lindqvist, 1938, p. 240) we examined to what extent arbitrary samples provide values which can be regarded as representative of the average concentration of vitamin A in the liver. Samples were taken from 20 necropsies: specimens of 30–50 mg. from places from which puncture biopsies may originate, and 10–20 specimens of about 10 g. of tissue from different parts of the liver. The results of the analysis of these specimens are seen from Table 1.

The differences between the results of the micro-analyses of specimens from the right lobe from the data from macro-analyses are all below ± 11 %, and their average difference is 4.65 %. The samples from the left lobe show, however, deviations up to 31.3 %. These findings agree with those of Lindqvist (1938). Since the left lobe constitutes about 20 % of the liver the errors committed by reckoning a micro-sample from the right lobe as indicating the average for the whole liver are small, and without committing significant errors one may reckon the deviation of biopsy micro-analyses from the average-vitamin A concentration of the liver to be c. 5 %, with c. 12 % as maximal value.

Table 1. Comparison between the results of macro-analyses and those of micro-analyses

(*d* indicates samples from right lobe of liver; *s*, samples from left lobe.)

	<i>E</i> _{1 cm.} ^{1%} from		Difference (results of micro-analysis minus result of macro-analysis) expressed as percentage of result of macro-analysis
	Macro-analysis	Micro-analysis	
1	0.057	0.063 (<i>d</i>)	+ 10.5
2	0.492	0.462 (<i>d</i>) 0.540 (<i>d</i>)	− 6.1 + 9.8
3	0.420	0.423 (<i>d</i>) 0.401 (<i>d</i>)	+ 0.7 − 4.5
4	0.107	0.112 (<i>d</i>) 0.090 (<i>s</i>)	+ 4.7 − 15.9
5	0.384	0.392 (<i>d</i>) 0.264 (<i>s</i>)	+ 2.1 − 31.3
6	0.038	0.040 (<i>d</i>) 0.037 (<i>s</i>)	+ 5.3 − 2.6
7	0.360	0.352 (<i>d</i>)	− 2.2
8	0.474	0.463 (<i>d</i>)	− 2.3
9	0.990	1.07 (<i>d</i>)	+ 8.1
10	1.32	1.28 (<i>d</i>) 0.96 (<i>s</i>)	− 3.0 − 27.3
11	0.114	0.107 (<i>d</i>) 0.135 (<i>s</i>)	− 6.3 + 18.4
12	0.260	0.271 (<i>d</i>)	+ 4.2
13	0.569	0.538 (<i>d</i>)	− 5.4
14	0.617	0.599 (<i>d</i>) 0.505 (<i>s</i>)	− 2.9 − 18.2
15	0.742	0.769 (<i>d</i>) 0.710 (<i>s</i>)	+ 3.6 − 4.3
16	0.323	0.327 (<i>d</i>)	+ 1.2
17	0.076	0.083 (<i>d</i>)	+ 9.2
18	0.115	0.108 (<i>d</i>)	− 4.9
19	0.249	0.238 (<i>d</i>) 0.203 (<i>s</i>)	− 4.4 − 18.5
20	0.466	0.472 (<i>d</i>) 0.484 (<i>s</i>)	+ 1.3 − 3.7
Mean	0.408	0.413 (<i>d</i>) 0.376 (<i>s</i>)	+ 0.85 ± 5.5 (<i>d</i>) − 10.60 ± 16.3 (<i>s</i>)

While the advantage of the puncture biopsy is that it can be repeated, its main disadvantage is its greater margin of error (cf. Table 1). Consequently the accuracy of an estimation of the liver depots based on a puncture biopsy must be inferior to that of one based on an operation biopsy, and especially to one based on necropsy material.

SUMMARY

A method is described for the quantitative determination of vitamin A in aspiration biopsies of the liver by the Carr-Price reaction. It provides a means of studying the storage in the vitamin A and carotenoids in man and larger animals on a much greater scale than before.

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The Application of the Silica Gel Partition Chromatogram to the Estimation of Volatile Fatty Acids

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The lower fatty acids, with the exception of formic acid, are so similar in their chemical properties that their quantitative separation and estimation by purely chemical methods have not proved possible. On the other hand, marked changes in physical properties occur as the series is ascended; for example, the volatility in steam increases and the partition coefficient between water and immiscible organic solvents decreases. It is not surprising, therefore, that the methods available for the estimation of these compounds in mixtures should be physical rather than chemical.

The distillation procedure of Duclaux (1874) and its numerous refinements make use of the observation that the volatile fatty acids distil in steam at different rates, and that in a mixture each distils at its own characteristic speed regardless of the presence of others. The partition method introduced by Behrens (1926), subsequently improved by Werkman (1930), depends on the fact that each fatty acid partitions itself between water and an immiscible organic solvent irrespective of the other acids present. Although both these methods, in the absence of more satisfactory ones, have found favour with the analyst, it should be emphasized that both have their limitations. They are inadequate for work with more than two acids, they are of little use by themselves for the identification of the constituents of a mixture, and neither achieves a complete resolution of even a binary mixture into its components. In consequence, the amount of each acid present is obtained by calculation rather than by direct assay. Indeed, until recently, no method has been available for the separation, identification and

estimation of the lower fatty acids in a mixture. Schick Tanz, Steele & Blaisdell (1940) introduced a procedure based on the fractional distillation, with an efficient column, of the azeotropes formed between the lower fatty acids and aryl hydrocarbons. In this manner they were able to separate a mixture containing formic, acetic, propionic, *n*-butyric and *iso*-butyric acids into its components. This represented a notable advance in technique, but the method is not quantitative and is scarcely suitable for routine analysis.

Lester Smith (1942) demonstrated that the silica-gel partition chromatogram, developed by Martin & Synge (1941), would separate a mixture of formic, acetic, propionic, *n*-butyric and *n*-valeric acids in chloroform into its component acids. Only compounds with differing partition coefficients between water and the organic developing solvent can be separated by this method. Martin & Synge (1941) incorporated in the gel an indicator of suitable pK for the location of colourless organic acids on the column, and for the separation of acetylated amino-acids they initially recommended methyl orange; this indicator was subsequently supplemented by anthocyanins (Gordon, Martin & Synge, 1943, 1944) which are not leached so readily as methyl orange. More recently Liddell & Rydon (1944) have introduced an azo dye, 3:6-disulpho- β -naphthalene-azo-*N*-phenyl- α -naphthylamine, with similar properties and the added advantage that it is readily prepared in quantity in the laboratory. Lester Smith (1942) found that methyl orange was of little use when the partition chromatogram was used for the separation of lower fatty acids, owing to lack of sensitivity, the