2. The increase in protein in the hypertrophy caused by hormones was confirmed for the levator ani muscle of the rat.

3. The percentage composition of the muscle was unchanged during the periods of hypertrophy and return to normal weight. With further loss of weight, the percentage of contractile proteins decreased and a corresponding increase in percentage of connective tissue occurred.

4. At 7 days after denervation a 36 % increase in absolute amount of sarcoplasmic protein accompanied by a 27 % increase in absolute amount of contractile protein was observed. At 45 days decreases in absolute amount of these two fractions of 45 and 54 % respectively had occurred.

5. There was no significant change in absolute amount of connective tissue during the period studied.

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# A Constituent of the Unsaponifiable Portion of Animal Tissue Lipids ($\lambda_{max.}$ 272 mμ.)

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The distribution of 7-dehydrosteroid (i.e. material showing ultraviolet absorption similar to that of provitamin D) in animal tissues has been studied by Glover, Glover & Morton (1952) and by Festenstein & Morton (1955). The small intestine is a relatively rich source in some species (e.g. guinea pig) but not in others.

In the course of their work Festenstein & Morton noted the presence in the intestines of horse and pig (but not sheep or ox) of a constituent of the unsaponifiable fraction which showed an absorption maximum at 272 mμ.

The selectively absorbing substance was concentrated by chromatography and characterized in terms of qualitative and quantitative ultraviolet absorption spectra in various solvents and by its adsorptive properties. Although horse intestine appeared to contain possibly 20 mg./kg. the yield of the richest concentrates was not very promising. A similar substance was found by Cain & Morton (1955) in horse liver but the investigation has been hampered by the presence of other very persistent congeners.

Some time later the '272 mμ. substance' was again encountered. Unsaponifiable fractions from various tissues of both normal and vitamin A-deficient rats were chromatographed on alumina and a 'rat 272 mμ. material' was obtained and identified with the 'horse and pig 272 mμ. substance'. More extensive study of various tissues from xerophthalmic rats and normal rats showed the substance to occur in kidney, liver, intestine and submaxillary gland, but to be absent from similarly separated fractions of bladder, testis and vagina. It is hoped in due course to comment upon the relative amounts of '272 mμ. material' in the tissues of control rats and vitamin A-deficient rats. It is clear, however, that the livers and kidneys of vitamin A-deficient rats are a fairly good source, and the same substance has been observed in hen liver and kidney. It also occurs (Kantiengar, Lowe, Morton & Pitt, 1955) in cockerel liver and kidney and in guinea pig liver (Leat, private communication). The present work records properties aiming at

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characterizing the 272 mμ. substance. It is proposed in what follows to use the abbreviation SA for the compound responsible for the absorption curve of Fig. 4 ($\lambda_{max.}$ 272 mμ., inflexion 330 mμ., flat peak near 410 mμ.).

## EXPERIMENTAL

### *Materials*

Fresh horse, sheep, ox and pig intestines were obtained from the Stanley Abattoir, Liverpool, and were treated in the manner described by Festenstein & Morton (1955).

Normal rat tissues were obtained from adult animals of the stock colony which had been reared on Animal diet no. 4 (British Extracting Co. Ltd., Bromborough Port, Cheshire) together with bread and milk. Vitamin A-deficient rats were produced by feeding weanlings the following diet: casein 18% (either (*a*) 'Low vitamin content' from Genatosan Ltd., or (*b*) 'Fat-free and vitamin-free' from British Drug Houses Ltd.), rice starch 65%, arachis oil 5%, Marmite 8%, salt mixture (Osborne & Mendel, 1913) 4%. When the rats exhibited loss of weight and xerophthalmia they were anaesthetized and killed by cardiac puncture, using a syringe and collecting blood.

### *Methods*

A few portions of intestinal tissue were ground with sharp sand and anhydrous $Na_2SO_4$ and the lipid was extracted with peroxide-free ether. The dry lipid was chromatographed on alumina weakened by treatment with water and the fractions were examined spectroscopically, using *cyclo*hexane as solvent. More usually, however, portions of tissue were subjected to alkali digestion and the unsaponifiable matter was extracted with light petroleum. The unsaponifiable fractions were chromatographed on alumina weakened with water (see below).

Rat tissues were treated as follows: to the moist tissue was added 60% (w/v) KOH (0·5 ml./g.) tissue and the mixture was heated on a water bath until it became homogeneous. Ethanol (2 vol.) was then added and boiling continued under reflux for 30–40 min., whereupon water was added and the mixture was extracted (five or six times) with ether (freshly distilled over reduced Fe). The combined ether extract was washed with water until free from alkali, dried over anhydrous $Na_2SO_4$, filtered through a sintered glass funnel and taken to dryness under $N_2$.

Chromatographic separations were carried out on alumina (Grade O. P. Spence and Co. Ltd., Widnes) weakened with 2% (v/w) water. Ether, light petroleum (A.R. b.p. 40–60°) and mixtures of these two solvents, were used as eluents. Both solvents were dried over Na wire and redistilled before use, the ether being redistilled over reduced Fe. Fractions were taken to dryness under nitrogen and examined spectroscopically.

## RESULTS

*Pig intestine.* The unsaponifiable material from pig intestine was fractionally crystallized and a solid fraction showing a weak maximum at 272 mμ. was chromatographed on 5% (v/w) watered alumina. 5% ether–light petroleum eluted a fraction (13 mg.) with $E^{1\%}_{1\,cm.}$ 272 mμ. 5·0. This was rechromatographed and 2% ether–light petroleum eluted 4 mg. of material with $E^{1\%}_{1\,cm.}$ 272 mμ. 7·6 and 4% ether–light petroleum eluted 4 mg. of similar material with $E^{1\%}_{1\,cm.}$ 272 mμ. 4·2. The absorbing entity showed no selective absorption in the region 300–500 mμ. and was seen on the column as a faint yellow band.

*Horse intestine.* A 12 ft. long portion of fresh intestine was carefully trimmed from fatty tissue adhering to the outside, most of the contents (hereafter referred to as mucus) were expelled by pressure along the length and the remainder was collected after longitudinal sectioning.

The fatty tissue, the mucus and the intestine proper were saponified separately and the resulting unsaponifiable fractions were crystallized from methanol to remove most of the cholesterol. The residual unsaponifiable matter was then chromatographed on 5% watered alumina in each case.

(*a*) *Mucus.* The unsaponifiable material from the first trial showed $\lambda_{max.}$ 270 mμ. $E^{1\%}_{1\,cm.}$ 5·4 and that from the second trial (0·11%) showed a similar broad band $E^{1\%}_{1\,cm.}$ 4·7.

On chromatography an orange zone passed down the column fairly quickly with light petroleum as the developing solvent. In one case a fraction (18 mg.) showing $E^{1\%}_{1\,cm.}$ 22·5 at $\lambda_{max.}$ 273 mμ. was obtained with 4% ether–light petroleum; in another experiment 2% ether–light petroleum eluted a substance with $\lambda_{max.}$ 268 mμ. and 4% ether–light petroleum a substance with the whole curve displaced ($\lambda_{max.}$ 273 mμ.). The most strongly adsorbed materials included cholesterol, 7-dehydrocholesterol and hydroxylated carotenoids. The least strongly adsorbed material showed selective absorption near 257 mμ.

(*b*) *Intestine.* The unsaponifiable material (0·24% of weight of tissue) showed $\lambda_{max.}$ 270 mμ. $E^{1\%}_{1\,cm.}$ 4·3.

On chromatography, light petroleum eluted a small amount of carotene together with material transparent to ultraviolet light; 2% ether–petroleum removed the remaining carotene and later gave a substance with $\lambda_{max.}$ 270 mμ. $E^{1\%}_{1\,cm.}$ 29 and $\lambda_{max.}$ 405 mμ. $E^{1\%}_{1\,cm.}$ 1·25 (probably SA).

In a second experiment 2% ether–light petroleum gave 0·14 g. of eluted material $\lambda_{max.}$ 271 mμ. $E^{1\%}_{1\,cm.}$ 20, and 4% ether–light petroleum 0·092 g. of material with $\lambda_{max.}$ 273 mμ. $E^{1\%}_{1\,cm.}$ 6.

(*c*) *Mesenteric fat.* The unsaponifiable fraction after chromatography showed clearly the presence of carotenoids but not of other substances showing characteristic ultraviolet bands.

(*d*) *Variation of '270 mμ. substance' along the length of the intestine.* Table 1 shows that the absorbing material is fairly evenly distributed and is not localized in the absorptive part of the gut.

Table 1. *Variations in unsaponifiable matter along the length of a horse intestine*

Values in parentheses refer to mucus, others to intestine.

| Length from stomach end (ft.) ... ... | 0–13 | 13–24 | 24–26 |
|---|---|---|---|
| Unsaponifiable (%) | 0·26 (0·15) | 0·23 (0·25) | 0·22 (0·21) |
| $E^{1\%}_{1\,cm.}$ 270–272 mμ. (gross, calc. on unsap.) | 3·3 (3·5) | 2·8 (3·1) | 3·7 |
| $E^{1\%}_{1\,cm.}$ 270–272 mμ. (calculated on unsap. but measured on best chromatographic fraction) | 1·2 (1·4) | 0·81 (1·5) | 0·94 |

Table 2. *Horse intestine lipids and unsaponifiable matter*

| | | Mucus | Intestine |
|---|---|---|---|
| Wet weight (g.) | | 190 | 500 |
| Lipid (%) | | 1·8 | 0·98 |
| Unsaponifiable matter, direct saponification (%) | | 0·15 | 0·23 |
| $E^{1\%}_{1\,cm.}$ 270–272 mμ.: | | | |
| (i) Measured on selectively absorbing fraction after chromatography of unsap. but expressed on wet weight | | 0·0038 | 0·0045 |
| (ii) Measured on two absorbing fractions after chromatography of lipid but expressed on wet weight | (*a*) | 0·0023 | 0·0067 |
| | (*b*) | 0·0012 | 0·0030 |
| | Sum | 0·0035 | 0·0097 |

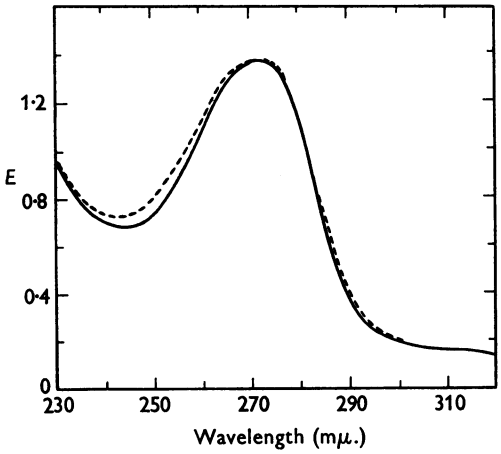



Fig. 1. 272 mμ. fractions from chromatography of horse intestine lipid or unsaponifiable fraction. ——, a lipid fraction (0·097%); - - - -, a fraction from unsaponifiable material (0·061%). Both fractions also show a weak band with $\lambda_{max.}$ 405 mμ. (0·97 and 0·61% respectively).

(*e*) *Occurrence of compound SA in intestinal lipids.* The experiments detailed below were undertaken to test the possibility that SA is a saponification artifact.

A 13 ft. length of horse intestine was taken from the stomach end, the mucus expelled by squeezing and flushing with water. The mucus and intestine were each divided into two portions, one of which was saponified and the other used for extraction of lipid. Both lipids were chromatographed and the absorption spectra of the fractions were measured (Table 2).

Fractions showing a well-defined absorption minimum near 245 mμ., a maximum near 272 mμ. and a much weaker peak near 405 mμ. were obtained by both methods (Fig. 1). The curves are identical in shape and it seems clear that the substance responsible is not a saponification artifact.

When the isolated lipid is saponified the yield of unsaponifiable matter confirms that obtained by direct digestion of mucus or tissue, but the 272 mμ. maximum is not shown. The substance responsible is labile to alkali (Table 4), but is protected in some way during the alkali digestion of tissue. This result eliminates the possibility that the absorbing material is a saponification artifact.

*Ox and sheep.* Chromatography of unsaponifiable material from ox and sheep intestine revealed no substance absorbing selectively at 270 mμ. Some carotene and 'xanthophylls' were, however, shown clearly in fractions from both mucus and intestinal tissue.

*Horse stomach.* The stomach and 10 ft. lengths of small intestine nearest to the stomach and caecum (including duodenum and ileum respectively) were obtained from the same animal. The stomach was cut open transversely and undigested food was removed. The oesophageal region (white and non-glandular) was separated from the darker glandular region where gastric juice is secreted (Sisson, 1943). The muscle layers could be peeled off easily using a scalpel. In each case the unsaponifiable fractions were chromatographed.

The '272 mμ. material' was found in all the tissues examined together with material showing appreciable absorption at wavelengths greater than 320 mμ. Table 3 summarizes the results, and Figs. 2 and 3 indicate how the duodenal fraction shows the best 270 mμ. curve, and the fraction from the outer layer of the oesophageal region the worst curve from the point of view of definition.

An unidentified carotenoid-like absorption spectrum ($\lambda_{max.}$ 430 mμ., inflexions 380, 410 and 460 mμ. in *cyclo*hexane) was found in fractions from horse mesenteric fat, horse duodenum, ox and sheep intestine. It was not found in extracts from horse ileum or stomach. Zechmeister & Tuzson (1935) state that carotene is the chief carotenoid of the horse, but also report what seems to be the same absorbing entity as that mentioned here.

*Rat liver.* The absorption spectra shown by the unsaponifiable matter obtained from the livers of both normal and vitamin A-deficient rats showed $\lambda_{max.}$ 270 mμ. In every case when these fractions were chromatographed on 2% watered alumina, 4% or 6% ether–light petroleum eluted a yellow fraction with $\lambda_{max.}$ 272–273 mμ., $\lambda_{min.}$ 237mμ., an inflexion about 330 mμ. and a very flat peak about 410 mμ. Compared with other tissues, rat liver proved a rich source of SA, fractions with $E^{1\%}_{1\,cm.}$ up to 170 at 272 mμ. being obtained after a single chromatographic separation.

*Rat kidney.* The unsaponifiable materials from the kidney of normal and xerophthalmic rats were characterized by absorption spectra with $\lambda_{max.}$ 270 mμ. When these fractions were chromatographed on 2% watered alumina, 4% ether–light petroleum always eluted a yellow band. The best fraction obtained was as follows ($E^{1\%}_{1\,cm.}$ in parentheses): $\lambda_{max.}$ 273 mμ. (160·0), $\lambda_{min.}$ 237 mμ. (42·5), inflexion, 330 mμ. (11·5).

*Rat intestine.* The unsaponifiable materials from intestinal tissue of both control rats (small but definite liver stores of vitamin *A*) and frankly vitamin A-deficient rats, contained small amounts of SA (order 0·2–0·8% of unsap.). In each case a very clear-cut separation of SA from other substances was obtained using 4% ether–light petroleum for elution.

*Rat submaxillary gland.* A maximum at 270 mμ. was characteristic of the unsaponifiable material from both control and vitamin A-deficient rat submaxillary glands. After chromatography on 2% watered alumina, 4% ether–light petroleum eluted a yellow zone. The best SA fraction obtained had $\lambda_{max.}$ 271 mμ. $E^{1\%}_{1\,cm.}$ 83·8 and the inflexion near 330 mμ. was shown.

*Other tissues of rat.* Examination of bladder, testes and skin from around the eyes of both normal and vitamin A-deficient rats and the vaginas of vitamin A-deficient rats by similar methods, failed to show the presence of SA.

*Hen liver.* The unsaponifiable matter from hen liver showed $\lambda_{max.}$ 271 mμ. Chromatography on 2% watered alumina again yielded a 6% ether–light petroleum fraction characterized by $\lambda_{max.}$ 273 mμ., the $E^{1\%}_{1\,cm.}$ was low (8·38) and definition poor.

*Hen kidney.* Again, the unsaponifiable fraction exhibited $\lambda_{max.}$ 270 mμ. and on chromatography, 4% ether–light petroleum eluted a yellow oil with $\lambda_{max.}$ 272–3 mμ. $E^{1\%}_{1\,cm.}$ 30·5.

Table 3. *Unsaponifiable matter from horse stomach and intestine*

| Tissue | % unsap. | $E^{1\%}_{1\,cm.}$ 270–272 mμ. gross on unsap. | $E^{1\%}_{1\,cm.}$ 270–272 mμ. after chromatography: On wt. of fraction | $E^{1\%}_{1\,cm.}$ 270–272 mμ. after chromatography: On wt. of original tissue |
|---|---|---|---|---|
| Stomach glandular region: | | | | |
| Muscle layer | 0·14 | 3·6 | 23 | 0·0012 |
| Outer layer | 0·12 | 1·9 | 52 | 0·0007 |
| Oesophageal region: | | | | |
| Outer layer | 0·145 | 1·0 | — | — |
| Intestine: | | | | |
| Duodenum | 0·185 | 2·6 | 48 | 0·0019 |
| Ileum | 0·19 | 3·1 | 41 | 0·0012 |

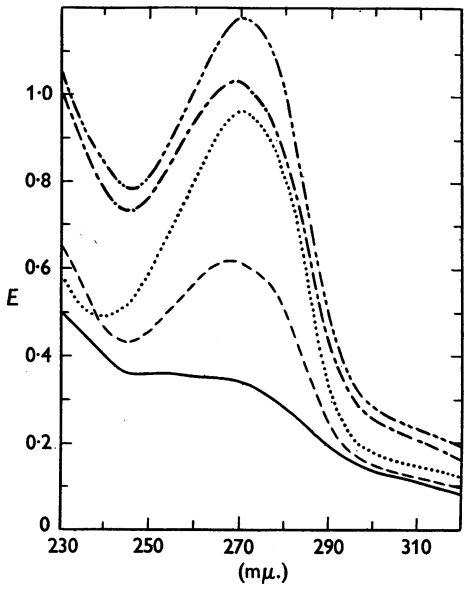



Fig. 2. ·····, Duodenum fraction 0·02%; – – –, ileum fraction 0·015%; –·–·, outer layer of stomach glandular region 0·04%; –·····–, muscle layer 0·05%; ——, oesophageal stomach region outer layer 0·018%.

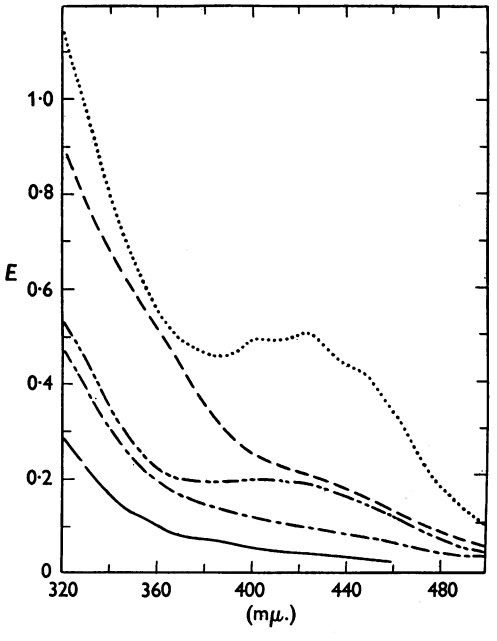



Fig. 3. ·····, Duodenum fraction 0·2%; – – –, ileum 0·15%; –·–·, outer layer of stomach glandular region 0·06%; –·····–, muscle layer 0·15%; ——, outer layer of oesophageal stomach region 0·07%.

Figs. 2 and 3. Absorption curves of '272 mμ.' fractions obtained after chromatography of horse unsaponifiable material from different tissues (note change of scale at 320 mμ.).

Table 4. *Absorption spectra of horse intestine and rat liver SA*

Tests carried out on best available fractions—see text.

| | Horse intestine sample (mμ.) | Rat liver sample (mμ.) |
|---|---|---|
| In *cyclo*hexane | | |
| $\lambda_{max.}$ | 272 | 272–273 |
| $\lambda_{min.}$ | 235–236 | 235–237 |
| Inflexion | 330 | 330 |
| $\lambda_{max.}$ (very flat) | 410 | 410 |
| $P (= E_{max.}/E_{min.})$ | 4·7 | 4·7 |
| Ratio $E_{272}/E_{330\,m\mu}$ | 13·7 | 14·0 |
| In other solvents | | |
| Light petroleum | 271–272 | 272 |
| Ethanol | 275 | 275 |
| Chloroform | 277 | 277–278 |
| ≃ 0·1 N Ethanolic HCl | 273·5 | 275 |
| ≃ 0·1 N Ethanolic KOH | Peak destroyed | Peak destroyed |

In conc. $H_2SO_4$, after 30 min. in dark at room temperature

| | (mμ.) | $E^{1\%}_{1\,cm.}$ | (mμ.) | $E^{1\%}_{1\,cm.}$ |
|---|---|---|---|---|
| $\lambda_{max.}$ | 315 | 300 | 313–315 | 337 |
| Inflexion | 360 | 160 | 355 | 182 |
| Weak $\lambda_{max.}$ | 480 | 6·0 | — | — |
| $E_{315\,m\mu}$ $H_2SO_4/E_{273\,m\mu}$ (*cyclo*hexane) | — | 1·54 | — | 1·99 |

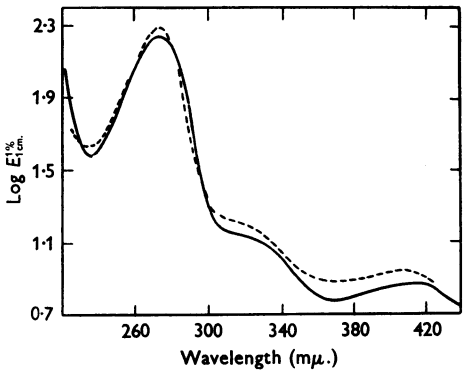



Fig. 4. Absorption curves in *cyclo*hexane for the best preparations of SA from: - - - -, horse intestine; and ——, rat liver. When SA is obtained in a state of established purity log $E^{1\%}_{1\,cm.}$ values can be converted to log $\epsilon$ (molecular extinction coefficient).

### *Concentration of SA*

The first experiments were made on horse intestine material. Little success resulted from attempts to concentrate the material by partitioning between pairs of solvents. One fraction (75 mg. $E^{1\%}_{1\,cm.}$ 70) was then shaken with five successive 5 ml. portions of cold methanol. The dissolved part (20 mg.) showed $E^{1\%}_{1\,cm.}$ 124 and the residue (59 mg.) $E^{1\%}_{1\,cm.}$ 58. The latter was, however, qualitatively the better in the sense that it had much less interfering absorption on the short wavelength side of the maximum.

Chromatography on 7% watered alumina showed the absorbing material to be slowly eluted with 2% ether–light petroleum. By combining fractions showing the highest persistence $P (= E_{max.}/E_{min.})$, and rechromatography, 10 mg. of a yellowish semi-solid material $\lambda_{max.}$ 272 mμ. $E^{1\%}_{1\,cm.}$ 195 (*cyclo*hexane) was obtained. This was chromatographed again and a very small amount of substance with slightly better persistence resulted. The spectroscopic properties of this material are compared with those of the best samples of rat liver SA in Table 4, while Fig. 4 compares their ultraviolet absorption spectra.

Table 5. *Fractions of SA obtained by rechromatography of two rich samples*

Solvent for absorption spectra, *cyclo*hexane.

Two samples bulked and placed on column:
(1) 17·5 mg. $\lambda_{max.}$ 272 mμ., $E^{1\%}_{1\,cm.}$ 172. $P = 3{\cdot}71$
(2) 16·9 mg. $\lambda_{max.}$ 272 mμ., $E^{1\%}_{1\,cm.}$ 176. $P = 3{\cdot}78$

Fractions obtained on chromatography.

| Fraction | $\lambda_{max.}$ (mμ.) | $E^{1\%}_{1\,cm.}$ | $P$ |
|---|---|---|---|
| (*a*) | 272–273 | 144 | 3·12 |
| (*b*) | 273 | 174 | 4·74 |
| (*c*) | 272 | 159 | 2·86 |

In view of the earlier experience with 'horse intestine SA', later attempts were made to concentrate the rat liver substance on watered alumina. It has been found that 2% water-weakened alumina is the most effective adsorbent for separation, SA being eluted with 4–6% ether–light petroleum mixtures. Using this procedure, two fractions totalling 34·4 mg. and with $\lambda_{max.}$ 272–3 mμ. $E^{1\%}_{1\,cm.}$ 172 ($P = 3{\cdot}71$) and 176 ($P = 3{\cdot}78$) were obtained. These were rechromatographed and three very viscous yellow samples of SA were obtained (*a*, *b* and *c*), all being eluted with 4% ether–light petroleum (see Table 5).

Although rechromatography did not increase the $E^{1\%}_{1\,cm.}$ value, it did raise the persistence of the best fraction (*b*) from 3·7 to 4·7. There are two possible explanations for this failure to raise the $E^{1\%}_{1\,cm.}$ value by rechromatography; first, the material may be nearly pure and secondly, it is just possible that SA is mixed with a non-absorbing material from which it is inseparable chromatographically. This point will be mentioned later in the discussion.

Substance SA failed to give an insoluble digitonide.

*Infrared absorption.* The absorption curves of *a*, *b* and *c* (Table 5) were recorded in $CS_2$ solution (Grubb-Parsons double-beam spectrometer). Samples *a* and *b* could not be distinguished; the principal maxima were at 2915, 1656, 1608, 1379, 1289, 1259, 1171, 1096, 1024, 980, 840, 741 cm.$^{-1}$. Sample *c* differed only from *a* and *b* in that the peak at 1171 had disappeared and maxima at 1156 and 1196 cm.$^{-1}$ were present. Fig. 5 shows the infrared absorption spectrum of fraction *b*.

*Analysis.* Fraction *c* contained no nitrogen and its molecular weight (Rast) was 430. Fraction *b*: C, 82·0; H, 10·25%; 0 (by difference), 7·75%. This corresponds with $C_{14}H_{21}O$ (requires C, 81·9; H, 10·3%; mol.wt. 410) which from the mol.wt. indicates $C_{28}H_{42}O_2$.

*Evidence for a second substance (with $\lambda_{max.}$ ca. 266 mμ.).* A large number of 'horse' preparations eluted before or with carotene were combined (0·43 g.) and chromatographed again on weakened alumina. Light petroleum eluted 0·34 g. of material absorbing weakly at 257 mμ. Two small fractions *l* and *m* were eluted when a little ether (5 and 10%) was added to the solvent ($E^{1\%}_{1\,cm.}$ 266 mμ. 33 and 64. $P = 1{\cdot}05$ and 1·19 respectively, solvent: light petroleum).

Both fractions were examined in conc. $H_2SO_4$ and showed a sharp band at 273 mμ. (*l*) also showed an inflexion at 355 mμ. which became a definite band in (*m*). When the ultraviolet curves are corrected for end-absorption the rise in the 355 mμ. band (in conc. $H_2SO_4$) is found to run exactly parallel to the increase in concentration of the 266 mμ. substance in (*m*).

Cholesta-3:5-dien-7-one shows $\lambda_{max.}$ 269 mμ. in light petroleum and $\lambda_{max.}$ 356 mμ. in conc. $H_2SO_4$ at the correct relative intensities for identifying this substance. Although this is not conclusive it seems probable that the fractions *l* and *m* contain about 1·5 and 4·5% respectively of the dienone or a closely related substance.

*Carotenoid.* A carotenoid-like substance with $\lambda_{max.}$ 430 mμ. (inflexions at 410 and 460 mμ.) was found in fractions from horse duodenum, mesenteric fat and ox and sheep intestine lipids. In all probability this is a breakdown product of β-carotene. In a forthcoming paper Dr J. Glover and Dr E. R. Redfearn will compare these bands with those of 10′-*apo*carotenal prepared by chemical methods from β-carotene. This compound could arise by oxidative metabolism of β-carotene in the gut and its presence is the likeliest explanation of the observations here recorded.

*Hydrocarbon from horse intestine.* A colourless hydrocarbon m.p. 57° (not sharp) was isolated from the combined light petroleum eluates (C, 85·2%; H, 14·6%; mol.wt. (Rast) 503). This was probably a normal paraffin ($C_{36}H_{74}$ requires C, 85·3; H, 14·7%; mol.wt. 506).

## DISCUSSION

Inspection of the spectroscopic characteristics, summarized in Table 4, and Fig. 4, together with the similar adsorptive properties, leaves little doubt that the horse intestine 272 mμ. material and rat 272 mμ. material are the same. Isolated from

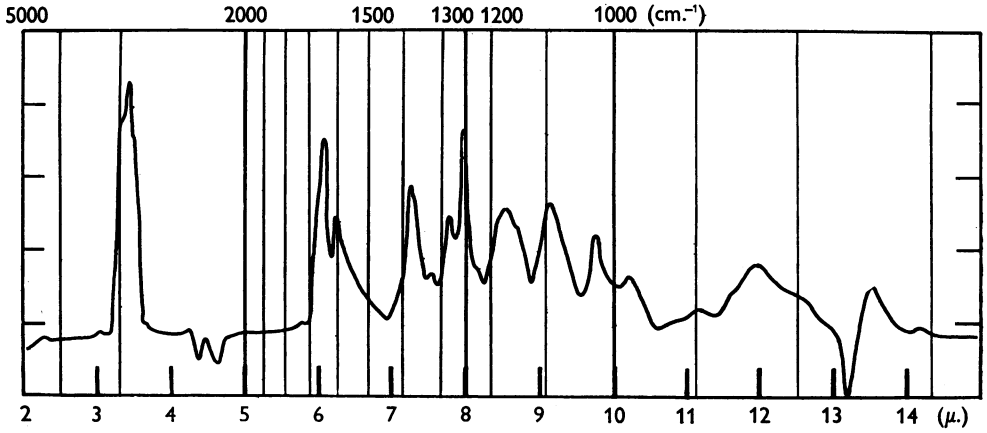



Fig. 5. Infrared absorption spectrum of fraction *b* (Table 5).

either source, SA shows the same position of $\lambda_{max.}$ in different solvents and the same loss of selective absorption in ethanolic KOH.

Fig. 4 shows that there is close agreement of the ultraviolet absorption spectra of the materials isolated from horse intestine and rat liver even when the spectra are subjected to the severe test of plotting log $E^{1\%}_{1\,cm.}$ against wavelength. There is, however, a discrepancy between the sulphuric acid spectra; the horse intestine material shows a weak $\lambda_{max.}$ 480 m$\mu$. which is not exhibited by the rat liver material. As the $E^{1\%}_{1\,cm.}$ values at corresponding points are slightly higher for the rat SA than for the horse SA in sulphuric acid, it is of interest to compare them at 480 m$\mu$. Here the weak maximum of the horse material has $E^{1\%}_{1\,cm.}$ 60·0 and the smooth curve of the rat material $E^{1\%}_{1\,cm.}$ 46·0. Since, at this point, the relative intensities are reversed compared with other portions of the spectrum, it seems that the horse preparation contains an impurity not found in the rat product. At this point, it should be mentioned that substances other than SA (to be discussed in later communications) occur in rat tissue which also show $\lambda_{max.}$ 315 m$\mu$. in sulphuric acid. Comparison of the ratio $E$ 315 m$\mu$. ($H_2SO_4$)/$E_{272\,m\mu.}$ (*cyclo*hexane) for the best fraction obtained from rat kidney (1·92) with that obtained from rat liver (1·99) suggests that these samples were free from such congeners.

The methods employed in the isolation of SA impose sharp limitations upon possible chemical structure. The fact that SA occurs in tissue unsaponifiable fractions implies that it could be a hydrocarbon, a steroid, a fat-soluble vitamin, a higher alcohol or perhaps a decomposition product. The absorption spectrum does not fit that of any known fat-soluble vitamin, and the fact that SA occurs in the tissues of xerophthalmic rats makes it unlikely to be a decomposition product of vitamin A. Analysis rules out a hydrocarbon, but the molecular formula points towards a steroid or similar lipid having two oxygen atoms per molecule. Compared with cholesterol, which is eluted from 2 % watered alumina by 8–10 % ether–light petroleum mixtures, SA must be relatively non-polar, because it is easily eluted from alumina of similar strength by 4 % ether–light petroleum mixtures. This fact suggests that the two oxygen atoms present in the molecule are more likely to be ketonic rather than hydroxyl groups, a conclusion supported by the infrared spectrum which shows no significant band in the region 2·68–3·22 $\mu$., the wavelength region for the stretching vibrations of the hydroxyl groups.

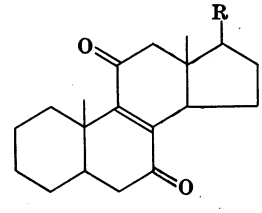
The dominant 270 m$\mu$. band in the ultraviolet absorption spectrum suggests a chromophore consisting of either three conjugated double bonds or, of a keto group conjugated to two double bonds or an ene-dione.

At this point, the infrared spectrum may be helpful. The band at 6·22 $\mu$. (1608 cm.$^{-1}$) implies either a conjugated or unconjugated (C:C) double bond [if conjugated the band would be expected at 6·21–6·33 $\mu$. (1610–1580 cm.$^{-1}$) if unconjugated the range is 6·06–6·25 $\mu$. (1650–1600 cm.$^{-1}$) Miller, 1953]. The peak at 6·04 $\mu$. (1656 cm.$^{-1}$) agrees with the stretching vibration of a conjugated ketonic group (Jones & Dobriner, 1949). Of the other principal bands shown in the infrared spectrum that at 3·43 $\mu$. (2915 cm.$^{-1}$) corresponds to —$CH_2$— stretching and that at 7·25 $\mu$. (1379 cm.$^{-1}$) to deformation of the methyl group of an *iso*propyl configuration, while that at 8·54 $\mu$. (1171 cm.$^{-1}$) agrees with the band due to the *iso*propyl group as a whole. The bands beyond 8·5 $\mu$. (1176 cm.$^{-1}$) are associated with vibrations subject to effects of interaction (fingerprint region) and interpretation of the bands occurring here is not obvious.

In view of the method of preparation of SA, and because of the absence of selective absorption in the region 1720–1760 cm.$^{-1}$, the strong band at 1259 cm.$^{-1}$ (7·94 $\mu$.) cannot be assigned to an ester; this band still requires explanation.

From the foregoing, SA is probably a steroid or similar lipid with a double bond conjugated with either one or two ketone groups. Furthermore, the steroid is likely to possess a cholesteryl side chain and angular methyl groups at $C_{10}$ and $C_{13}$ in order to account not only for the molecular formula but also the *iso*propyl and —C—$CH_3$ groups indicated in the infrared spectrum.

The ultraviolet absorption spectra of steroids have been summarized and classified in a recent comprehensive review by Dorfman (1953). The number of chromophores corresponding with selective absorption at 270–275 m$\mu$. is not large (see Table 6), and the nearest behaviour to that of SA is shown by compounds containing the 8(9)-ene-7:11-dione chromophore.




It is of interest to note that the carbonyl-stretching band for steroids with the 8(9)-en-11-one configuration is at 1660 cm.$^{-1}$, whereas for steroids with the 11-one group only it is at 1710–1704 cm.$^{-1}$ (Jones & Herling, 1954). The band at 1656 cm.$^{-1}$ in the infrared spectrum of fraction *b* (Table 5) has been assigned to the carbonyl groups. Unfortunately all the known substances which possess this chromophore also contain substituents at position 3.

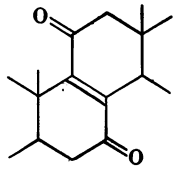
Table 6. *Steroids showing* $\lambda_{max.}$ *near* 270 $m\mu$.

See Dorfman, 1953.

| Chromophoric grouping | Substance | $\lambda_{max.}$ ($m\mu$.) | $\epsilon_{max.}$ | $E^{1\%}_{1cm.}$ |
|---|---|---|---|---|
| 6:8(9)-Diene | *iso*Dehydrocholesterol | 275 (ether) | 5300 | 138 |
| 2:4-Diene | Cholesta-2:4-diene | 267 | 6300 | 171 |
| | | 275 | 6300 | |
| Enols of 2:3-diketones | Cholestane-2:3-dione | | | |
| | cholest-3-en-3-ol-2-one | 272 | 5000 | 125 |
| | cholest-1-en-2-ol-3-one | 270 (ethanol) | 8500 | 212 |
| Enol of 6:7-diketones | 3$\beta$-Acetoxy-cholestane-6:7-dione | | | |
| | Cholest-5-en-6-ol-7-one or cholest-7-en-7-ol-6-one | 275 (ethanol) | 10700 | 258 |
| 8(9)-Ene-7:11-dione | (i) 5$\alpha$-Pregn-8(9)-ene-7:11:20-trione-3$\beta$-ol acetate | 268 | 6607 | 171 |
| | (ii) Cholest-8(9)-ene-7:11-dion-3$\beta$-ol benzoate | 269 | 6310 | 122 |
| | (iii) Methyl 3$\alpha$-acetoxy-7:11-dioxochol-8-enate | 272 | 8128 | 177 |
| | | 271 (ethanol) | 7079 | 154 |
| | (iv) 3$\beta$-Acetoxyergosta-8:22-diene-7:11-dione | 270 (ethanol) | 8710 | 186 |
| | | 266 (*iso*octane) | 9360 | 200 |

For 8(9)-ene-7:11-dione chromophore see also Fieser & Herz (1953). Fieser, Huang & Babcock (1953) and Fieser, Schneider & Huang (1953).

Furthermore, the tendency to report spectra in the steroid field in terms of $\lambda_{max.}$ and $\epsilon_{max.}$ only, means that inflexions are often not recorded in the literature. However, the curve for 3$\beta$-acetoxyergosta-8:22-diene-7:11-dione (Heusser, Eichenberger, Kurath, Dällenbach & Jeger, 1951) shows a minimum near 235 $m\mu$. and apparently an inflexion in the region of 330 $m\mu$. The evidence is consistent with the presence of this chromophore in SA.



## SUMMARY

1. A substance (SA) with an absorption peak at 272 $m\mu$. (in *cyclo*hexane), an inflexion near 330 $m\mu$. and a plateau near 410 $m\mu$. ($E^{1\%}_{1cm.}$ 180, 13 and 8 approx. respectively) has been obtained by chromatography on alumina of various animal tissue unsaponifiable fractions.

2. SA is present in pig intestine, horse stomach and intestine, rat liver, kidney, intestine, and submaxillary gland whether from vitamin A-deficient or normal rats. SA was not found in unsaponifiable fractions from ox or sheep intestine or from bladder, testis and vagina of vitamin A-deficient rats.

3. SA is not very strongly adsorbed on alumina, it contains two oxygen atoms (no hydroxyl) and a possible formula is $C_{28}H_{42}O_2$ (molecular weight observed 430); it is alkali-labile, particularly so in the purer state. From the polarity, infrared and ultraviolet absorption and probable empirical formula, SA could be a steroid with a chromophoric grouping 8(9)-ene-7:11-dione.

4. Horse intestine unsaponifiable matter contained small quantities of material slightly less polar than SA with $\lambda_{max.}$ 266 $m\mu$. (possibly cholesta-3:5-dien-7-one) a carotenoid (probably 10′-*apo*-carotenal) and a higher paraffin (m.p. 57°, mol.wt. 503).

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# The Determination of Calcium in Serum by Flame Photometry

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Since 1948, when Riehm first demonstrated the feasibility of the direct flame-photometric method for the determination of calcium in dilute solutions, this principle has been applied to analysis of soil (Stanford & English, 1949), milk (Keirs & Speck, 1950) and serum (Severinghaus & Farabee, 1950). In the following year further reports of serum calcium determinations appeared from Leyton (1951) and from Mosher, Itano, Boyle, Myers & Iseri (1951), followed by those from Kapuscinski, Moss, Zak & Boyle (1952), Zak, Mosher & Boyle (1953), and Powell (1953). While the present account was being written, direct determinations on diluted serum have been described by Winer & Kubins (1953), Chen & Toribara (1953) and by McIntyre (1954). Several of the American authors used modified spectrophotometers, and in nearly all cases sera have been prepared for analysis by procedures involving oxidation, precipitation of calcium, or removal of sodium. The comparative advantages of simply diluting with water are evident. Although many forms of photometer, most of them elaborate, have been described, the desirability of using simple and relatively inexpensive equipment still remains.

The determination of calcium in blood by flame photometry presents peculiar difficulties. The concentration of calcium is small but that of sodium and potassium large, while the concentration range of calcium occurring in human sera is narrow, so that considerable accuracy is required. The normal range in human serum is 9·0–11·5 mg. Ca/100 ml., and even grossly pathological conditions extend this ambit only to the limits 4–16 mg./100 ml. If precipitation and other pretreatment of the sample are to be avoided, the serum must be diluted at least tenfold, so that the final concentration of calcium is about 1 mg./100 ml. This dilute calcium solution emits very little light from a flame. The use of an interference filter permits the partial isolation of the calcium oxide red bands (approx. 6200 Å), provided that didymium glass is used to reduce the excess of sodium light also emitted. In an acetylene–air flame, maximum output of energy in the calcium bands is ensured, and the intensity of this light, although very low, can be measured either by the use of a photoconductive cell (Schwarz, 1948) as described below, or by means of photomultiplier apparatus.

The construction and use of an internal standard photometer which has proved satisfactory for the determinations of calcium, sodium and potassium in diluted serum and other material are described below.

## EXPERIMENTAL

### *Description of photometer*

The general lay-out of the instrument is shown diagrammatically in Fig. 1.

*The burner unit.* Acetylene is burnt after admixture with the air-borne sample, and no atmospheric air enters the system. The brass Meker-type burner is shown in section in Fig. 2. The jet (*A*) is a standard welding component (British Oxygen Co. Ltd., London, type DH, 2 cu.ft./hr. i.e. 940 ml./min.), and is screwed into position over a fibre washer (*B*). The top-plate (*C*), of brass, is perforated as shown. Acetylene at a pressure only slightly above atmospheric enters at *D* and the air-mist through *E*. The burner is clamped in a boss attached to a plate (*A*, Fig. 3) bearing a light-trap (*B*) and bolted to a plate (*C*) of similar outline brazed to the chimney-tube (*D*) which it supports. This tube has diametrically opposed apertures (*E*, *E'*) for exit of light, and a smaller hole (*F*) for insertion of a taper for lighting.

*Gas control and atomizer.* Both acetylene and air are taken from industrial cylinders fitted with two-stage regulators. The acetylene regulator is fitted with an outlet needle-valve which is used as on-off control to avoid readjustment of the succeeding valve. Fine needle-valves incorporated in the photometer are used for both gases. Acetylene from its