# THE α-GLYCERYL ETHERS

by

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## GENERAL INTRODUCTION

We are concious, in presenting a review of the work of others, and of our own, that the greatest difficulty of scientific life to-day lies not only in its manifold complexity but in the fact that the world of our time has not yet mastered the means of intercommunication**.

We have tried to put together a summary of the relevant parts of an investigation which has gone on for many years. The details will be published in due course in the journals devoted to clinical medicine, radiology, biology and biochemistry.

In order to attempt to present a coherent whole, we have thought it desirable to put forward hypotheses which are our own, and which we all must make from time to time, and which we, like others, must be prepared to discard if and when necessary in the light of future knowledge.

In the study of the unsaponifiable fraction of many fish oils certain factors are found occurring in varying proportions; these are:

(1) Sterols.

(2) Terpenoid hydrocarbons, mainly squalene.

(3) Glyceryl ethers or esters thereof.

Little biochemical or biological attention has been attracted to groups (2) and (3) in so far as man is concerned, and although our purpose is to consider the nature, occurrence, and effect of the last group with reference to the higher mammal, we feel it wise to consider some aspects of squalene as preliminary to our main discussion.

## SQUALENE

*Occurrence*

Squalene, a hydrocarbon, $C_{30}H_{50}$, is found present in liver oils of many of the Elasmobranchs and was originally thought to be linked to the presence of the glyceryl ethers of group (3) above. LOVERN[1] however pointed out that, as this hydrocarbon was absent from the liver oil of the gray dog fish and the rat fish (both of which are rich in the glyceryl ethers) the argument was untenable.

Although present in 5% of the unsaponifiable fraction of adult human serum, it has been suggested by DEUEL[2] that it plays an important part in embryological devel-

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** See "Symposium of Utilisation of Recorded Knowledge" (*Federation Proc.* 16 (1957) 703).

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opment. It has been shown to be a constituent of the vernix caseosa[3] in man (but not of the placenta) and of ovarian dermoid cysts[4] and of depot fat of women[5].

Since the initial discovery of squalene in fish liver oils (TAUJIMOTO, 1906), it has also been shown to occur in the vegetable kingdom, especially in the Palestinian, Turkish, Tunisian and Spanish olive oil[6] and in yeast fat[7].

*Biological significance*

Heller[8] suggests that squalene of biological origin may serve as a precursor of cholesterol, whereas the synthetic hydrocarbon does not, the difference being attributable to stereo-specificity. However, cholesterol is absent from the vegetable kingdom and it seems to us more probable that squalene furnishes isoprenes which could well be used in the synthesis of the carotenoids and of the Mg-porphyrin component of chlorophyll, the skeletal similarity of which SZENT GYÖRGYI has recently pointed out, or for the isoprenoid side chains of the vitamin K analogues. Indeed, one might speculate with WERTZEL[87] that such a hydrocarbon chain represents an initial stage of embryonic or vegetable fat synthesis via olefin production and subsequent oxidation to fatty acid. The carotenoids are carried in the $\beta$-lipoprotein fraction of plasma and the $\beta$-carotene–vitamin A synthesis is carried out in the intestinal mucosa. The evidence suggests strongly that the placenta does not allow passage of material carotene to the foetus, nor is there evidence to prove that vitamin A as such freely passes the placental barrier[10]. At birth the vitamin A level has suffered a "precipitous drop" from which, after a few days, it will recover[9]. It would appear that blood carotene levels begin at birth at minimal levels, rise to a maximum at 12 months and then fall to adult level.

GETZ[103] records that carotene conversion to vitamin A appears to be blocked in tuberculous patients, the alcohol not being adequately released from the ester. The administration of crude cod liver oil has a significantly greater effect in rectifying this than does synthetic vitamin A.

Finally it must be recorded that the levels of plasma carotenes and vitamin A fall after the fiftieth year and that throughout adult life the levels in women are lower than those found in men[11] even after saturation by oral administration, whether they are estimated as the alcohol or the ester.

## THE α-GLYCERYL ETHERS

*General characteristics*

The three glyceryl ethers are as follows:*

| | |
|---|---|
| chimyl alcohol | α-hexadecylglyceryl ether. |
| batyl alcohol | α-octadecylglyceryl ether. |
| selachyl alcohol | α, 9-octadecenylglyceryl ether. |

The last of these is known to exist in two forms, the *cis* and *trans* isomers, the *cis*, so far as is known, occurring only in nature. As happens with the two similar forms of oleic acid (Table II) the *cis* form is of lower melting point, and the molecule is far less extended.

* For the sake of brevity these will be referred to as AGE.

*References p. 272–274*

TABLE I

GLYCERYL ETHERS

| | | |
|---|---|---|
| Chimyl alcohol | ether of cetyl (palonityl) alcohol (*n*-hexadecanol) and glycerol. m.p. 60.5° | $CH_3(CH_2)_{15}CH_2O\cdot CH_2\cdot CH(OH)CH_2OH$ |
| Batyl alcohol | ether of steryl alcohol (*n*-octadecanol) and glycerol. m.p. 70–71° | $CH_3(CH_2)_{16}CH_2\cdot O\cdot CH_2\cdot CH(OH)CH_2OH$ |
| Selachyl alcohol | ether of oleyl alcohol (*cis*-9-octadecanol) and glycerol. m.p. 8°. The *trans* form is also known m.p. 48°–49° | $CH_3(CH_2)_7\cdot CH{=}CH\cdot(CH_2)_8\cdot O\cdot CH_2\cdot CH(OH)\cdot CH_2(OH)$ |

This *cis-trans* isomerism is readily changed *in vitro*, and there is some[42] evidence that it may be changed under biological conditions *in vivo*. Similarly, it is known that enzyme systems exist, especially in multilocular fatty tissues, which are capable of interconverting oleic and stearic acid.

It is known[43] that these substances form eutectic mixtures and some care must be exercised in determining the melting points and in the application of these results. For example, the naturally occurring *cis*-selachyl alcohol is recorded[42] as having a melting point of 18–19° whereas other sources state—as in our experience—that it occurs as an oil at ambient temperature.

TABLE II

STEREOCHEMICAL ISOMERISM OF OLEIC ACID

| *Oleic acid* | *Elaidic acid* |
|---|---|
| $CH_3\cdot(CH_2)_7\cdot CH$<br>$\|\|$<br>$COOH\cdot(CH_2)_7\cdot CH$ | $CH_3(CH_2)_7\cdot CH$<br>$\|\|$<br>$HC\cdot(CH_2)_7\cdot COOH$ |
| *cis*,9-octadecenoic acid | *trans*,9-octadecenoic acid |
| Crystallises in 2 forms m.p. 13° and 16° | m.p. 43.7° |

As might be expected, the double linkage of selachyl alcohol is exhibited as fluorescence under the U.V. lamp (365 mμ).

As the three substances are each α-glyceryl ethers, it would be supposed that each would show optical rotation. This is the case, and has been shown to be true also of the synthetic homologues produced by BAER AND FISCHER[42, 44, 45]. It has been observed that batyl alcohol in high concentration shows a negative rotation, gradually diminishing to disappear at 10% concentration. Further dilution shows dextrorotation[46].

Evidence has been brought forward[47] that in naturally occurring fish oils these AGE are present as fatty acid esters, each of the remaining hydroxyl groups of the glycerol being esterified with a higher fatty acid.

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Lovern[17] has pointed out that the AGE occur in fish oils whose fatty acids show a subnormal degree of unsaturation and has suggested that they represent a hydrogenation of the glyceride molecule, representing reduction of an ester carbonyl group. BAER AND FISCHER[45] have suggested that they are formed from the reductive splitting of the appropriate C—O bond of the acetal ring (and removal of phosphoric acid) from acetal phosphatides. More recently it has been found[46] that the aldehydes of the plasmalogens do exist in the *enol* form, which is, in effect an ether linkage to the glycerol.

The AGE were originally found in the liver oils of certain Elasmobranchs and were isolated from the unsaponifiable fractions thereof. However, even in this group, their appearance is almost haphazard. For example, the greatest concentration found in the basking shark *(Cetorhinus maximus)* occurs as 9% of the unsaponifiable fraction from the flesh as against 1.6% of the unsaponifiable fraction of the liver oil.

Of four vegetable oils examined, the AGE were found[18] present in Californian tung oil *(Aleurites fordii)* alone.

The AGE were recovered from body cavity fat, intestinal fat and liver oil (but not from subcutaneous fat) of the rat *(Rattus norvegicus)* and the domestic hen, the esters also being present in egg yolk[12]. They have been found present in bone marrow of cattle[13] and the spleen of pigs[14]; in erythrocytes[16] and arteriosclerotic arteries in man[15*] but not in human liver[12]. One is left with the impression that the tissue common to all recorded sources of these AGE in the animal kingdom is mesenchymal in origin.

*The mesenchymal origin of AGE*

During the embryological development certain mesenchymal cells give rise to lipoblasts, the fore-runners of fat cells. The evidence points to the fact that these cells are already destined for this purpose while they are yet morphologically identical with other cells of embryonic connective tissue.

Fat globules do not appear in these cells in man until the fourth month of development, nor do foetal glycerides assume importance in animals before the middle of gestation, after which they gradually increase. Prior to this the phospholipid content is highest and non-phospholipid fat is present as free and unesterified fatty acids[20]. The relative percentage of unsaponifiable lipid decreases proportionately as gestation proceeds.

Histologically, two types of fat cell are recognisable and two types of adipose tissue by macroscopical appearance—"white" and "brown" fat. WELLS[49, 50] reviewed the position in great detail and suggests that peri-renal fat in man (*cf.* interscapular fat) is the homologue of the brown fat of the hibernating mammal. WERTHEIMER AND SHAPIRO[51] have recorded the enzymatic differences between white and brown fat and have shown, for example, that "brown fat" contains phosphoglucomutase while "white" fat does not.

The fat within the cell may, in fact, store either hormones or vitamins or other fat soluble products of physiological or pathological metabolism, but the enzyme make-up of the cell would seem to be a function of its specific developmental (mesenchymal) prototype, which as we have pointed out before, is determined before the cell

* It is interesting to record here the finding of β-carotene in atheromatous plaques[52].

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is morphologically differentiated. Hence, although fat is not shown as such before mid-term, this is no evidence that the enzymatic function of the cell itself is not active.

The developmental mesenchymal cell may, under certain conditions, undergo proliferation and form a tumor, known as a "hibernoma". These were reviewed by JENNINGS AND BEHR[52] and probably occur more frequently than the literature would suggest, and are all almost certainly non-malignant. Unfortunately, detailed chemical analyses of the fat in these tumors have not been carried out. The unsaponifiable content has been shown to be 12% of total lipoids[53], 26 and 24% in two cases as against 9 and 10% of unsaponifiable fractions in two subcutaneous lipomata[54]. In one case unstained sections showed star shaped clusters of needle like crystals within a cell boundary, which crystals showed birefringence and were assumed to be of cholesterol[54]. PEARSE[55] summarises the difficulties of such diagnosis and quotes among the "battery of reactions" including birefringence and fluorescence a positive Schiff reaction as occurring with plasmals under certain conditions. The AGE have a habit of crystallising from anhydrous acetone in rosettes of microscopical proportions in very dilute solutions, to most beautiful "daisy heads" from those more concentrated. JENNINGS AND BEHR record the presence of intracytoplasmic bodies giving positive staining with sudan black and periodic acid Schiff reagents in their case report of hibernoma. Both of these histochemical reactions would be positive for AGE. It is within the bounds of possibility that AGE might be detected by histochemical means as occurring in such tumours.

The birefringent crystals shown in excellent microphotographs[54] are similar not only to cholesterol, but also with those of batyl alcohol. They certainly could be separated and identified under micro column conditions from the fat.

As will be shown later, the evidence suggests that the AGE are in cows and man associated with prenatal development, and the precise identification of the chemical nature of hibernoma tissue could give assistance in showing if they do in fact initially arise in mesenchymal tissues.

Before we leave the embryological sphere, we must, since to this we must return, consider the development of the amniotic fluid and of meconium. Amniotic fluid is of foetal and not maternal origin and it is surprising how very little is known to-day about its chemical constitution and the relation thereof to development. The water content is reabsorbed and replaced every three hours[63]. Of this fluid the foetus swallows some 500 ml in 24 hours[66] and passes into it small quantities of urine. Neither the sugar, uric acid or protein content bears any relation to that of the mother. The nature of the constituent proteins have been examined in detail[59] and the nature of the contained pigments, iron and copper recorded by BEVIS[60]. Very little is known about the enzyme or hormone content. JOHNSON[61] considered that a proteose was present and more recently[62] VENNING has described the presence of aldosterone. Stearic acid and cholesterol is also present. The meconium or "sterile faeces" of the foetus contains cetyl alcohol (see DEUEL[56]) and stearyl alcohol, which cannot therefore be the result of fermentation reactions of intestinal organisms, and it would be reasonable to assume their presence in the lipoids of the amniotic fluid.

Consideration of fibrocystic disease of the pancreas has led to the opinion that the physico-chemical structure of meconium is under control by pancreatic enzymes, which arise from the secretory granules in the exocrine cells. These appear about the

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fourth month of gestation, and their appearance is evidence of structure and not of function. Normally these granules release their enzymes slowly or not at all unless food enters the gut and stimulates the upper small intestinal mucosa to release the appropriate hormones which control the nature and constitution of pancreatic juice. The highest enzyme concentration is elicited by milk stimulation.

It is possible that the secretory granules might be stimulated by some substance contained in the amniotic fluid which is swallowed, and it is quite easy to show that meconium contains no inhibitor to a proteolytic enzyme such as chymotrypsin. However, DOLLAR *et al.*[86] have shown that the calf pancreas does not furnish any pancreatic amylase until some weeks after birth.

We have shown that these AGE are associated with major degree of saturation in the fatty acids in the unsaponifiable fraction. There is, in some species at any rate, association between squalene and the AGE whether this be coincidental or not; we shall show that the AGE are present in neonatal perinephric calf fat and are also present in human meconium and we have shown above the evidence that the component alcohols of the AGE are present in liquor amnii and the vernix caseosa of man and the inference is that the AGE will be present in the liquor and we shall consider later the evidence of clinical application of the liquor amnii to wound healing alongside our own experimental and clinical findings of biological extracts and purified preparations of the AGE.

A table is appended to clarify terminology of fatty acids relevant to this discussion (Table III). *Cis/trans* vaccenic acid, isomeric with oleic acid was found by LASSER to exist in blood[98] and a further isomer, the so-called G acid of GABR[99] was isolated from $\beta$-globulin fraction of human plasma. Both of these seem to be of physiological importance, in that they both have gut-stimulating properties. The interconversion of stearic and oleic acids and the highly specific properties of the isomers of the latter show very briefly the importance of such processes to the individual.

As described above[18] the occurrence of these AGE is almost haphazard in different species. Squalene rich oil has been found with no other undigested material in shark stomach[100] but in the land animal it appears in each report of examined faeces. Since the intestine is concerned in the metabolism of higher alcohols and, for example in the $\beta$-carotene – vitamin A reaction, it would not be unreasonable to think that the gut mucosa was concerned in AGE synthesis. This would give some reason for their presence, which we report here, in meconium. There is no recorded evidence of AGE being found in the human liver. They are constantly recorded as being present in those tissues where mesenchymal elements are constantly regenerated, such as marrow, spleen and intestine. There is no recorded evidence for their presence in sperm, but LOVERN *et al.*[101] have given results for lipid analysis of ram semen and the methods used for elution of the columns used encouraged us to look for AGE in unsaponified fractions eluted by ether. This will be reported later. Again, a free hydrocarbon, probably heptacosane, was present.

MARTINOLI[18] has published analyses of perinephric fat in calves showing that this in the neonatal animal is scanty and the component fatty acids are more saturated than at later stages in development.

We would summarise this section by saying that the AGE are found present in those tissues containing cells of mesenchymal origin, from which tissues cells are continually shed into a body cavity.

*References p. 272/274*

TABLE III

NOMENCLATURE OF FATTY ACIDS

| | | | |
|---|---|---|---|
| | | *Saturated fatty acids* | |
| Lauric | $C_{12}H_{24}O_2$ | *n*-dodecanoic | $CH_3(CH_2)_{10}\cdot COOH$ |
| Myristic | $C_{14}H_{28}O_2$ | *n*-tetradecanoic | $CH_3(CH_2)_{12}\cdot COOH$ |
| Palmitic | $C_{16}H_{32}O_2$ | *n*-hexadecanoic | $CH_3(CH_2)_{14}\cdot COOH$ |
| Stearic | $C_{18}H_{36}O_2$ | *n*-octadecanoic | $CH_3(CH_2)_{16}\cdot COOH$ |
| Arachidic | $C_{20}H_{40}O_2$ | *n*-eicosanoic | $CH_3(CH_2)_{18}\cdot COOH$ |
| | | *Unsaturated fatty acids* | |
| *Mono-ethenoid* | | | |
| Oleic | $C_{18}H_{34}O_2$ | *cis*, 9-octadecenoic | $CH_3(CH_2)_7\cdot CH=CH(CH_2)_7\cdot COOH$ |
| Vaccinic | $C_{18}H_{34}O_2$ | *cis/trans*, 11-octadecenoic | $CH_3(CH_2)_5\cdot CH=CH(CH_2)_9\cdot COOH$ |
| Gadoleic | $C_{20}H_{38}O_2$ | 9-eicosenoic | $CH_3(CH_2)_9\cdot CH=CH(CH_2)_7\cdot COOH$ |
| *Di-ethenoid* | | | |
| Linoleic | $C_{18}H_{32}O_2$ | 9,12-octadecadienoic | $CH_3(CH_2)_4\cdot CH=CH\cdot CH_2\cdot CH=$ $=CH\cdot(CH_2)_7\cdot COOH$ |
| *Tri-ethenoid* | | | |
| Linolenic | $C_{18}H_{30}O_2$ | 9,12,15-octadecatrienoic | $CH_3\cdot CH_2\cdot CH=CH\cdot CH_2\cdot CH=$ $=CH\cdot CH_2\cdot CH=CH\cdot(CH_2)_7\cdot COOH$ |

TABLE IV

| *Mesenchymal tissue* | *Sheds* | *Cavity* | *AGE also found in* |
|---|---|---|---|
| Bone marrow | erythrocytes | Vascular system tissue space | erythrocytes |
| Spleen | lymphocytes etc. | Vascular system tissue space | no evidence, except in splenic tissue |
| Intestinal lining | continually regenerating and shedding cells into lumen | Gut | present in faeces |

## EXPERIMENTAL

### *Introduction*

Since the AGE are found in fats which show a higher content of saturated triglycerides than is normal, it might be reasonable to suppose the AGE to be soluble therein and hence to be separable with them by fractional crystallisation from acetone. BEAR AND FISCHER[18] report that the AGE can be separated from ratfish liver oil by standing the oil in a refrigerator, filtering off the deposit and recrystalling from acetone the batyl and chimyl alcohols. GUIDETTI AND CASTOLDI[78] have recorded separation of saturated triglycerides by repeated acetone crystallisation at room temperature.

The physical constants for the pure selachyl and batyl alcohol used as reference substances in this experimental work were found to be as follows (Table V).

Crystals of batyl alcohol show well marked birefringence when examined with polarised light.

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TABLE V

*Typical analysis selachyl alcohol*

| | | |
|---|---|---|
| Iodine No. (Wijs) | 73.0 | Theoretical 74.0 |
| % Glyceryl ether (as selachyl alcohol) | 98.4% | Periodate oxidation and dimedone assay for formaldehyde |
| Hydroxyl value (mg KOH/g selachyl alcohol) | 316 | Theoretical 327<br>Our assay on fractionated acetate (WITTER thesis U.B.C., 1948) 314 |
| Refractive index $n_D^{20}$ | 1.4680 | TOYAMA $n_D^{20}$ = 1.4691<br>BAER synthetic $n_D^{22}$ = 1.4672 |
| Specific gravity $d_{20}^{20}$ | 0.923 | BAER synthetic $d_4^{22.5}$ = 0.923 |
| Melting point | 10.5° | Literature m.p. for *d*-selachyl alcohol is 17–19° |
| F.F.A. (as oleic) | 0.94% | |
| Saponification value (mg KOH/g selachyl alcohol) | 8.2 | |

*Typical analysis batyl alcohol*

| | | |
|---|---|---|
| % Glyceryl ether (as batyl alcohol) | 99.5% | Duplicate average periodate oxidation and dimedone assay for formaldehyde |
| Hydroxyl value (mg KOH/g batyl alcohol) | 315 | Theoretical 326 |
| Melting point | 68.3–68.7° | Literature 71–72° |

The discrepancy in m.p. of selachyl alcohol has been referred to above. The specimen of batyl alcohol shows slight fluorescence, possibly due to traces of selachyl alcohol being present.

*Methods*

*A.* (1) Perinephric fat from neonatal calves was put through the homogeniser, washed in cold acetone and dried. The dried residue was dissolved in warm petroleum ether and the insoluble portion discarded. The solvent was evacuated in vacuo and the residue recrystallised repeatedly from hot anhydrous acetone to 15° until the separated material showed a melting point of approximately 56° and an iodine value of 8–12. This gives a white freely running powder with a greasy smell, saponification number 198. This has been repeated many times with minor variations of these three values (m.p., iodine number and saponification number).

Continued crystallisation from acetone leads to lowering of the iodine value and raising of the melting point which indicates loss of AGE components among other possible "impurities" remaining in the saturated triglycerides.

(2) The unsaponifiable fraction was then separated according to the standard method of the British Pharmacopeia[21] and dried to constant weight and redissolved in light petroleum and fractionated on an alumina column (B.D.H.), the alumina being previously heated to 200° for two hours.

(3) Elution was carried out according to SWAIN[22]

| | |
|---|---|
| Light petroleum | to remove hydrocarbons, |
| benzene | to remove monohydric alcohols, |
| ethyl ether | to remove dihydric alcohols, |
| methanol | to remove remaining unsaponifiable material. |

(4) Identification was carried out as follows: From the diethyl ether fractions, material was obtained on evaporation of the solvent which, on repeated recrystallisation from anhydrous acetone, separated in white plates—melting point 68°.

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As suggested by EMMERIE *et al.*[23] esterification with phthalic anhydride and pyridine gave a clear solution on addition of water. On acidification and extraction by ether, the esters will pass as sodium salts into 5% sodium bicarbonate solution.

Further, as suggested by EMMERIE[24], periodate oxidation of material and chromatographic investigation on paper gave spots with identical $R_F$ values obtained with pure batyl alcohol on Feulgen Schiff staining.

An infra red spectrogram of the powder prepared from neonatal calf fat has kindly been furnished by Professor GUIDETTI (Fig. 1).

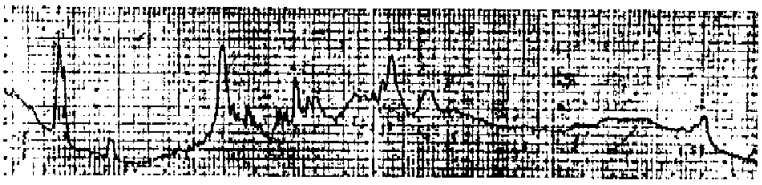

Fig. 1

*B.* The same procedures were applied to a pooled sample of human meconium.

*C.* The unsaponifiable fraction of rat fish liver oil was dealt with in a similar manner.

For animal experimentation it should be sufficient to refrigerate this oil and subject the deposit to fractional crystallisation from hot acetone. Recrystallisation at room temperature yields a preponderance of crystals with m.p. of 66° and to —5° with a preponderance of crystals with m.p. of 60°, which crystals are a mixture of the AGE in which batyl alcohol predominates in the first instance, and in the second instance there is considerably more chimyl alcohol.

This part of the work under report has been carried out by one of us (J.B.) whose opinion it is that it is a mistake to purify this product too far. It may be that the presence of small quantities of a substance which is probably selachyl alcohol, as exhibited by fluorescence, acts as a "primer".

The AGE were recovered as batyl alcohol from both the neonatal calf fat described above and from meconium.

We are indebted to Dr. JOHN HOLMBERG for confirmation of the presence of the AGE in the neonatal calf fat. His quantitative analysis of the specimen of powder supplied showed the presence of 5% of these glycerol ethers. We have not as yet identified the esters.

The powder prepared from neonatal calf perirenal fat does not show the presence of either P or S. Analysis of several samples give N as present (average of 5 estimations) as 0.05 mg% dry weight. Dissolved in hot methanol, the powder gives a faint Molisch reaction, and after treatment with periodic acid gives a positive Schiff reaction.

### *Animal experiments*

One of us (J.H.M.) has investigated the use of a (calf) extract on wound healing of irradiation injury.

These injuries were produced in Wistar rats on both hind limbs, one series being treated with the powder containing AGE and one with a talc placebo containing antibiotic.

The details are recorded in MAISIN's illustrations (Figs. 2 and 3). This work which has been in progress for three years will be reported in extenso elsewhere.

The effect on wound healing is very clearly demonstrated and what perhaps is most striking is that it is essentially the beginning, or stage I, of wound healing which is clearly initiated in the AGE treated section.

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CONTROLS

TREATED BY APPLICATION OF TALCUM POWDER AND ANTIBIOTIC (astreptine 1%)

| *Rats* ♀ | *DFP* | *KV* | *mA* | *Filter* | *Field* | *Time* | *Dose* |
|---|---|---|---|---|---|---|---|
| 1/A, B, C, D, E | 4 | 90 | 4 | 0 | 2 cm diam. | 19″ | 14.820 r |
| 2/A, B, C, D, E | | | | | | 14″ | 10.920 r |

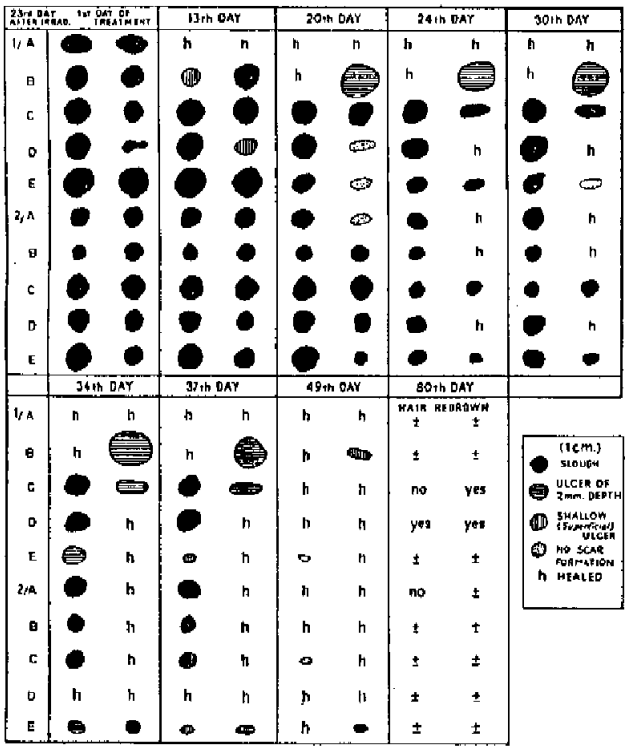


Fig. 2. Wounds treated with the placebo.

Experiments are now in progress to show if any prophylactic effect against similar irradiation damage can be demonstrated.

MAISIN has also shown previously[83, 84] that cystein and other like preparations will protect, in near toxic doses, against irradiation injury but that the hazards of genetic damage and carcinogenesis remain.

In view of the report[86] that post irradiation treatment can modify genetic damage, experiments are in progress to investigate the effect of glyceryl ethers in duplicate with the reported techniques. Duplicate experiments using AGE of fish oil extraction are in hand.

*References p. 272/274*

CONTROLS

TREATED BY POWDER CONTAINING ALKOXY GLYCERYL ESTERS

| *Rats* ♀ | *DFP* | *KV* | *mA* | *Filter* | *Field* | *Time* | *Dose* |
|---|---|---|---|---|---|---|---|
| 1° A B C D E | 4 | 90 | 4 | 0 | 2 cm diam. | 19″ | 14,820 r |
| 2° A B C D E | | | | | | 13″ | 10,140 r |

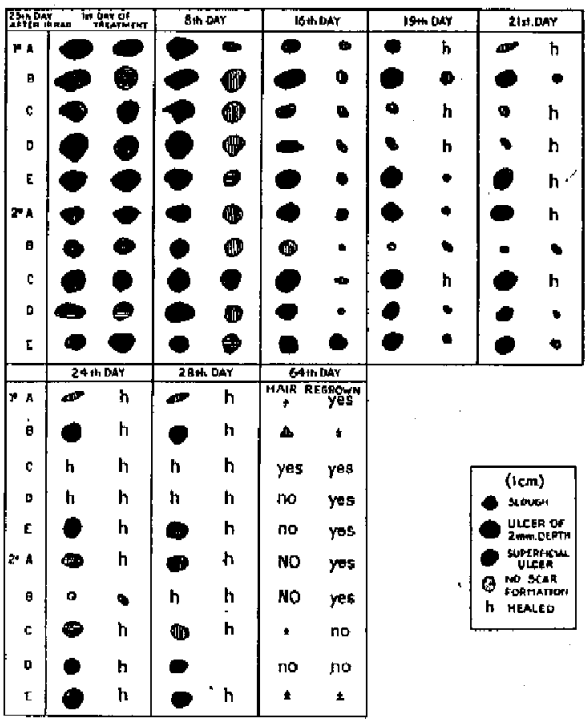


Fig. 3. Wounds treated with AGE.

The question naturally arises as to the precise nature of the di-acyl esters occurring biologically. To this end we are using AGE fractionated from ratfish oil solely by temperature control. Ratfish oil is unique in that there is virtually no triglyceride content.

*Human Clinical Trials*

The use of the AGE has been investigated in four types of wound damage*.

*(1) Non healing septic wounds in elderly patients*

*Male aged 60.* First admitted in 1948 complaining of haematuria. He had several small papillomata of the bladder. Three further admissions in 1948 for fulguration of the papillomata.

* It is not proposed in this publication to do other than report initially on typical case histories in each group.

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In June, 1956, bleeding recurred and he was re-admitted. Cystoscopy revealed a neoplasm of the bladder too extensive for transurethral fulguration and, on the 26th June, he had a right ureteric transplant into the sigmoid colon, and, on the 24th July, a left ureteric transplant into the sigmoid colon, with closure of the sigmoid colon, and left inguinal colostomy through the incisional wound.

He made a rather stormy convalescence and, on the 11th August, he had a transverse colostomy as the previous colostomy had retracted into the abdomen.

The infected, gaping inguinal wound, about 12 cm long was healing badly. The local application of AGE for one week definitely initiated granulation of the wound.

The patient continued to have intermittent fever and became progressively more drowsy and unable to take food.

The wound healed remarkably well, except for a small sinus at the lower end, but his general condition deteriorated and the blood urea became progressively higher and he died in uraemia on the 30th October, 1956.

*Comment.* An unhealed, gaping, septic wound of some three months' duration healed by granulation almost completely following local AGE application in some three weeks despite oncoming terminal uraemia and without other further surgical or antibiotic treatment.

### *(2) Non healing wounds associated with surgical treatment of malignant tumours*

*Female aged 40.* Patient presented with tumour of left breast and involvement of axillary glands. Histologically, the tumour was shown to be an adenocarcinoma. Radical amputation was carried out. Three weeks later the wound, extending 15 inches downwards from the axilla was seen with gaping edges about 5 cm apart with a thin purulent discharge.

*Treatment*: For two days the wound was dressed with gauze soaked in a saline solution of chymotrypsin (20 mg%) twice daily. The enzyme was allowed to stay in contact with the wound surface for two hours and then a Eusol dressing was applied.

AGE dissolved in warm sterile arachis oil (100 mg in 2.5 ml) was injected intramuscularly daily. The injection is painless and is not followed by any reaction.

After four days the wound is clean and fresh granulation tissue can be seen. The lower part of the wound shows approximation of the edges in about 4 cm. Injections of AGE continued and dry dressing with dusting of AGE powder applied daily.

Six weeks after the amputation was carried out the wound is healing well.

On the 63rd day patient is discharged with wound healed throughout and the treatment stopped.

The patient has attented for routine follow-up examinations over two years and the wound has not broken down since healing.

*Comment.* A long unhealed and infected surgical incision following amputation of breast for malignant disease was seen to start healing after enzymatic debridement and AGE administration.

### *(3) Non healing septic wounds associated with surgical treatment of fractures*

*Male aged 68.* Compound comminuted fractures of both legs, 7 in. above the ankle. Five weeks later the bones of the right leg were plated. The patient remained in bed with both legs in plaster for one month. Consequent upon fever, the plaster casing of the left leg was opened and a gangrenous wound was seen about 9½ inches long with heaped up edges between which the ends of the ununited tibia and fibula could be seen. Injections of AGE material were given as described previously together with the local application of the AGE containing powder daily. Within five weeks, it was considered possible to begin a skin graft. The plate on the right leg had to be removed during this period and when the plaster was taken off the right leg it was seen that a stubborn wound was present. Local application of the AGE containing powder was begun immediately and three weeks later "roll down" tube grafts for both legs had been begun. No further orthopaedic surgery was considered necessary and X-ray of the bones showed that union was beginning. Daily injections were continued for periods intermittently throughout the next year during the stage of plastic repair. The final condition two years after the initial injury, the patient is able to walk without mechanical aid and has complete healing of both legs, a photograph of which is shown (Fig. 4).

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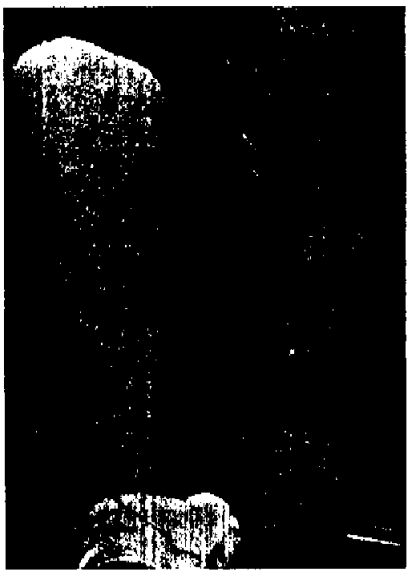

Fig. 4

*Comment.* It is a tribute to plastic surgery that such damage can be made good.

The interesting features of this case are:

(1) the almost immediate granulation of the gangrenous wounds which received no other local treatment; and

(2) the bone union which took place without further orthopaedic surgery.

The administration of AGE seems to stimulate all tissues of mesenchymal origin if these are abnormal.

*(4) Non healing wounds associated with X-ray injury*

The local application of the AGE accompanied by intramuscular injection of them in solution appears to be followed by almost immediate beginning of healing of radium or *X*-irradiation burns.

*Comment.* The cases include direct lesions (epitheliomata) and skin injuries due to deep irradiation of underlying structures such as malignant affections of the ovary.

The one striking failure in this particular series was in a case of osteoclastoma of the lower end of the tibia in a young man of 20, to which bone deep *X*-ray treatment had been given and over which bone the skin had broken down. The patient had returned to work on a walking calliper and was otherwise fit and well.

*(5) Healing of normal wounds (industrial or accidental) burns in normal healthy factory employers*

No effect whatever has been observed in the use of the fractions in the treatment of accidental burns in otherwise fit individuals. If anything, local application of the

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AGE fractions of any derivation has inhibited the healing process [86], and the exhibition of these materials was stopped.

*Toxic effects observed in these clinical trials*

Injections have been made with the AGE dissolved in arachis oil. The saturated triglycerides are virtually insoluble in arachis oil, but are slightly soluble in de-acidified olive oil and hence this solvent perforce had to be used and the suspension warmed before injection. No adventitious reaction, other than an occasional sensitivity to the solvent used, has been seen.

It is a practical alternative to prepare a stabilised emulsion for this purpose, and it has been found possible to do this with the help of Tween-80. Such emulsions have been maintained for fifteen months under laboratory conditions.

Water soluble preparations have been prepared as the sodium salt of the phthalate ester.

Prolonged administration by mouth of the powder prepared according to the suggestion of GUIDETTI have been carried out, with no signs of toxicity. The use of galactose as excipient has been successfully tried. It must be noted that about ten % of normal volunteers complain of nausea after ingestion of the oral capsules containing this powder. Such symptoms are by no means so common with AGE concentrates.

Electrophoretic (paper) examination of the serum of patients receiving AGE by intramuscular injection show increase of serum protein bound lipoids in the beta region. This, of course is not proof that such increase is due to the administered AGE.

BROHULT has reported[91] on the results of prolonged oral administration of glyceryl ether concentrates, prepared by JOHN HOLMBERG, from shark liver oil, and used in her department at the Radiohemmet, Stockholm.

Further clinical detail of our work will appear elsewhere.

## DISCUSSION

The pattern or somatic tissue is maintained, not because somatic cells inherently lack the power of movement, but rather that, under normal circumstances, they do not use it. As ABERCROMBIE[87] has pointed out, as long as the somatic cell is surrounded on all sides by similar cells each bearing a recognition symbol, that cell will remain in its appointed place in the community pattern.

The effect of wounding is to deprive a group of cells of their neighbours' recognition symbols, and these cells then show sudden swelling and develop their power of amoeboid movement. Two tissues only will be discussed briefly in this paper, liver and skin. Whether this movement initially be the mesothelial cells of the liver, or the fibroblasts of subcutaneous tissue, stage I of wound healing is essentially movement of somatic cells into an adjacent area which has already been invaded by contents of blood vessels whose walls, under the influence of the permeability factors[88], have allowed large protein molecules and cells to escape. The migration of cells takes place after a short latent period of 24 h and this stage I is the first step of an ordered sequence of events whose processes are determined by the "curious time cycles which govern them"[89].

DUNPHY *et al.*[70] have shown that, during this lag phase, there is accumulation of polysaccharide in the wound area, and with it synthesis of collagen. BULLOUGH[20] has shown that there is a very great in-flux of glucose during this period.

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Thirty-six h after wounding, there is a burst of mitotic activity in the cells of the epidermal sheet which has migrated into the wound, the cells of which have been halted by the "recognition symbol" of their fellows. This mitotic activity is energised, and in part controlled, by the great in-flux of carbohydrates, as glucose, into the wound tissues.

The third stage is that of contracture and of bringing together and firmly binding together the edges of the wound. ABERCROMBIE *et al.*[71] have put forward the idea that the mesenchymal cells of the regenerating connective tissue have this function, and GLUCKSMAN[29] has said that this is brought about by the fibroblasts of dermal origin, the fibre formation of which requires the uptake of $-SO_4$ ions. KENT[30] has described the metabolic pathways involved in the provision of sulphate from the storage depots of cartilage. This stage III begins on the fifth or sixth day of repair[72] and is marked by the formation of collagen, the polysaccharide decreasing rapidly from the fifth to the ninth day, and then more slowly. SLACK[73] has shown that intraperitoneally injected labelled sulphate is taken up for this purpose, and PRUDDEN *et al.*[31] have shown that the application of powdered tracheal cartilage applied to the wound increases the tensile strength of the repair.

Having briefly considered the essential details of the phenomena of wound healing, we must discuss physiological variations and pathological interference.

It has been known since the days of SPALLANZANI[74] that the shape of the wound affected the rate of healing. It was left to DU NOÜY[32] to describe the regularity of the rate of healing and to deduce an equation from which the anticipated time for healing could be calculated[33]. Whatever his metaphysical deductions may have been, his observation, data and calculations are refreshing to read. His conclusions are borne out by BILLINGHAM AND RUSSELL[75] and could be applied to the animal results of one of us (J.H.M.) as recorded in this paper.

It also became obvious to DU NOÜY that, when he moved outside his terms of reference (young soldiers in World War I) a correction factor had to be introduced in terms of increasing age of the patient, which is again borne out by BILLINGHAM AND RUSSELL.

But another factor has recently been brought to light by WEISS[34]. WEISS has shown that there is an age in development *before* which the repair of wounds does not take place and, further, that although the migration of the epidermal sheet does not take place before that date, the second stage, that is mitosis, does take place and results in the proliferating cells "pile up" at the wound edges. Hence, the first and second stages must be under different control. The crucial date is the twelfth day of (chick) incubation. It would be interesting to know if this is an "all or none" stimulation, or whether the repair of these wounds decelerates, or accelerates with increasing days of embryonic life.

"It might well be", so writes a special correspondent to the *Brit. Med. J.* "that the process of detachment of normal living epithelial cells in the body was commoner than hitherto expected".

Clinical observation will remind us that the first stage of wound healing is inhibited by sepsis, *X*-ray irradiation and sometimes by malignant disease among other conditions.

Starvation or physical fatigue will slow down the rate of mitosis but starvation will not inhibit this. There is an inhibitor to the permeability factors.

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Contracture is inhibited by protein starvation (relieved by giving methionine) administration of cortisone (possibly relieved by oestrogen) ionisation radiation and vitamin C deficiency.

EGER[78] has shown that repair and regeneration of allyl alcohol induced liver damage is accelerated, and that some protection against such damage might be given by spleen extracts, cysteine and methionine, and he also showed that fully grown female rats were less susceptible to such damage than were fully grown males, which he attributes to the anabolic effect of oestrogenic hormones, in which opinion he found support from GYÖRGYI[77].

EGER[79] has reported that Wistar rats, irradiated prior to the administration of allyl alcohol, show less liver necrosis than do control rats receiving irradiation. This is quite contrary to his expectations.

Interest has centred on various uses for the amnion, its contained fluid and insoluble material. Originally it was thought of as being responsible for preventing the foetus from "sticking" to the sac wall, and hence was used empirically to avoid peritoneal adhesions after abdominal operations[63, 81].

HOCHULI[46] has reviewed the effect of amniotic fluid injected into experimental animals after hepatectomy and shows the observed effect of swelling in the cell nuclei preceding the mitotic phase, which was not present in untreated controls. There was no acceleration of liver regeneration, but this unexplained "swelling" was consistently noted. Repair stimulation of irradiation and other cellular damage is discussed.

ZOLLINGER[86] reports that inunction of amniotic fluid stimulates epithelial proliferation in the skin of old rats, and shows that this is associated with increased cell mitosis and swelling of the nuclei and nucleoli, control animals not showing a similar picture. In a second paper[87] he reports on the use of this material in surgical (skin) wounds in man, which are recorded as healing more quickly and leaving smaller scars.

These papers then show no evidence of stimulation of liver regeneration in otherwise normal young animals, but do record stimulation of skin repair in older animals and in damaged (irradiation) animals and in surgical wound repair in (presumably) sick humans.

There is much similarity in this report with our own observations on the use of AGE. It might be that amniotic fluid contains the necessary fraction of AGE for incorporation into a more complex molecule, and hence that the therapeutic effect of amniotic fluid in wound healing extracts are similar to these brought about by the glyceryl ethers.

It is well to review very briefly here the known pharmacological effect of AGE administration. SANDLER[87] studied the erythropoietic effects of yellow bone marrow extracts as well as those of batyl alcohol, and was of the opinion that their administration was followed by reticulocyte response. This was taken further by BROHULT AND HOLMBERG[26] who investigated the effect of the AGE on cases of irradiation leucopaenia, with successful result. BROHULT continued this investigation at the Radiohemmet and has reviewed the results of four years clinical experimentation. She has found that there is an optimum dose level, beyond which it is not wise or useful to go[81]. EDLUND[25] has recorded the protective effect exerted by the AGE in mice receiving total body *X*-irradiation. He records the beneficial effects of such treatment, but again concludes that over-dosage increases the lethality of the irradiation.

It has been shown by EVANS *et al.*[101] that the administration of batyl alcohol to

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cattle suffering from bracken poisoning (a fatal condition of bone marrow damage similar to that produced by radiomimetic drugs) is followed by clinical recovery and restitution of the white cell and platelet count to normal.

EMMERIE *et al.*[28] have shown that a fraction of cod liver oil separated by column chromatography inhibits the growth of tubercle bacilli *in vitro*. This fraction contains batyl alcohol. It is also recorded that a fraction in crude cod liver oil concentrates[100] not associated with the vitamin A is favourable to healing of lesions in tubercle patients.

In surveying the field of irradiation damage and possible prophylaxis and/or treatment of such damage, LORENZ AND CONGDON[88] and later HEUWIESER[89] discuss various humoral factors and tissue extracts which have been used. This is again reviewed[90] and now the proof of the transfer of viable marrow cellular elements[40] is given, which has led up to the establishment of techniques for maintaining such marrow cells *in vitro*. BILLEN[41] extended such maintenance time to 21 days. Nevertheless, it seems to us that the use of such marrow cells would be complicated by the necessity of blood grouping possible recipients. MILLER[91] showed that injections of embryonic liver tissue (which can be also maintained *in vitro*) prevents death from acute radiation, and since embryonic liver is functionally haematopoietic tissue, we wonder if such cells as would develop therefrom would not carry blood group antigens which are not present in early (human) foetal erythrocytes. But the proof that blood forming elements are thus transferable does not of itself disprove that humoral factors might be of some use in the treatment of less severe radiation injury.

The effects of radiation, that is penetrating radiation of the *X* or gamma rays would seem to be partly cellular causing chromosome breakage, which if kept open in the absence of necessary metabolic enzyme systems, might lead to chromosomal fragments rejoining in the "wrong" sequence, leading to exchange linkages; and partly humoral. These latter effects are partly due to enzyme damage, partly due to uncontrolled depolymerisation of hyaluronic acid ground substance (by opening N-acetylglucosamine linkages). In addition, MÜLLER[92] has described humoral factors produced by ionising radiation which inhibit cell division. That is to say that serum of irradiated rabbits (but not serum irradiated *in vitro*) added to standard medium decreases the growth rate of *E. coli* to a highly significant degree ($P<0.01$), the decrease in growth rate being, in fact, a prolongation of the initial stationary phase. Incubation of the inhibitor-containing serum with cells of calf thymus depresses the level of this inhibition. Lastly, this ionising radiation affects the structure of water. Water has two crystallisation temperatures, at 0°, when we see and feel a rigid structure, ice, and the second between 30° and 40° where its tetrahedral lattice organisation finally disappears. The two protons of the $H_2O$ molecule can link with two other similar molecules by their H-bonds while the electron pair link up with two protons from two other water molecules leaving one $H_2O$ molecule linking four others in a tetrahedral lattice, which is permanent and rigid below 0°. This system tends to obtain in a less rigid form up to 30°-40°.

Biological processes in warm blooded mammals must be adjusted to this basic phenomenon which can be envisaged as been severely disrupted by gamma radiation leading to peroxide formation.

Dissolved molecules are now thought of as being surrounded by what FRANK AND EVANS christened "icebergs"[93] and molecules involved in energy transfer are known

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to be stabilised in their triplet state by —SH groups, and would very readily lose such stability from ionising radiation. It would be facile to build an imaginary iceberg structure round a molecule of ground substance (which, as we have seen above is depolymerised by ionising radiation) and then to re-organise the functional structure by atebrin in high dilution, for atebrin is capable of long life phosphorescence in frozen solution in high dilution (0.0005 *M*).

There remains one more consideration. We have seen that wounds in chick embryos lack either the ability to respond to the stimulus necessary for the first stage of wound healing, that is to say that the cells of mesenchymal origin fail to migrate, or that this stimulus is lacking. Further, we have seen that this inability to migrate lasts up to the 12th day of developmental life. AREY[38] in discussing the development of haematopoiesis writes, "In all the locations about to be mentioned, haemopoiesis is made possible by the *freeing* of mesenchymal cells which then serve as proliferative stem cells". (Italics ours). We therefore sought the advice of our colleague[34], Dr. CHUBB, of the Houghton Poultry Research Station, for authoritative information on the date in chick development when erythrocyte development in bone marrow takes place. He informs us that this has been researched upon and reviewed[35-37]. In the embryo the yolk sac is the main haematopoietic organ in the early stages. The liver and spleen have slight erythropoietic activity between the seventh and ninth days but both these organs are more concerned with the production of lymphocytes and granulocytes; erythropoiesis is taken up by the bone marrow at the twelfth day of incubation.

The cells of mesenchymal origin are freed in two places on the same day. The yolk contains trace quantities of the AGE as we have recorded above, sufficient for early needs, and we think that beginning synthesis of embryonic fat by the mesenchyme in the marrow affords these substances for the "freeing" of mesenchymal tissue or alternatively that these substances are formed by mesenchymal cells elsewhere for the benefit of the organism as a whole.

Hence it would seem that the continued production of AGE for the land-based mammal would be unnecessary and even embarrassing and, with the formation of triglycerides, we should expect the titre of AGE gradually to decrease as indeed we have shown to occur after birth in the calf. We should expect to find that gross excess of these AGE to the adult animal would be reflected in pathological signs in the adult, and we have recorded the fact as recorded in the literature that over-dosage of the AGE is not desirable. EMMERIE *et al.*[33] in fact state that the AGE are toxic to the guinea-pig, and death is caused by over-production of connective tissue elements within the peritoneal cavity. We have shown that in man the exhibition of the AGE in normal cases with normally healing wounds (burns) is not followed by any acceleration of the healing process.

Finally, if our argument is sound, we should expect to find that damage by irradiation in sublethal doses would be less when the AGE content were normally greatest, or that the effect thereof would be in some way mitigated. Two examples only are cited.

It has been shown that irradiation of the neonatal rat[95] is followed by a reticulocyte crisis. "This remarkable crisis", conclude the authors, "is one of the peculiar reactions of the haematopoietic system of the new born rat which, so far have been little studied".

STEWART *et al.*[96] in a current survey of malignant disease in children consider

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that maternal irradiation of the abdomen for diagnostic purposes, which amounts to total body irradiation of the foetus in utero "shortly before birth" increases the risk to the child of developing leukaemia or cancer thereafter. While we do not wish to put an interpretation on the quotation which is not statistically proved as yet, we feel that the matter is so serious that we include this paragraph in the hope that such figures will eventually be broken down to show if pelvimetry by *X*-ray examination might be accompanied by a lowered risk of such sequelae if the examination were made in any one month of gestation.

## CONCLUSIONS

The AGE were thought to be curious phenomena, half-glyceride half-wax, individual to certain deep sea mammals and fish of the Pacific Ocean. To-day their presence has been identified in whales, the vegetable kingdom, the common domestic egg and in man.

In land-based animals the AGE are found associated with mesenchymal tissue and especially with those cells which continue most closely to carry out their original function.

It may seem a curious fact that the AGE are said to occur as diacyl esters and yet that these naturally occurring esters have not been identified. It is not known for certain if these AGE exist as breakdown products from, or building stones for synthesis of, more complex molecules; nor is the site of their formation, in either event, known.

The evidence suggests that their storage is the function of the multilocular granular fat cell. They are found in highest concentration in those depots where adult unsaturated fat is lowest, or in those organs or tissues where constant cell production is maintained.

It might be thought probable that such storage would be necessary for the hibernating animal to enable the constant production of essential cells such as blood elements to continue during prolonged sleep. It is suggested that this storage would be in the specialised brown fat. In nonhibernating animals it would be reasonable to seek them in the homologues of the brown fat, that is in the perinephric or interscapular depots.

We suggest that it is more likely that the AGE are incorporated into a complex molecule and that these molecules are acetal phospholipids and that this incorporation occurs in the intestinal wall, the unwanted AGE being excreted in the faeces or meconium, and, if in meconium, then in amniotic fluid, and in vernix caseosa.

We have shown that the AGE used in animal experiment or in clinical treatment in man stimulate the first stage of wound repair, though our own results have in no way proved how this action takes place. We have shown that, after application to a wound, the AGE are slowly absorbed and we have slight indication that the β-lipoproteins are increased after their administration.

We suggest that the AGE contribute to the synthesis of one or more of the Darmstoffe, so named by VOGT[103], and thus assist in the "sodium pump" moving Na and K-ions across the cell membrane against the concentration gradient, and we suggest that this may be the factor initiating the swelling and subsequent of the cell.

Nevertheless, we have seen no evidence of similar physiological effect on acceleration of wound healing consequent upon exhibition of the AGE in cases in which normal wound healing had already begun.

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SUMMARY

The glyceryl ethers have been identified in neonatal calf fat and methods of extraction and identification are described. They are found only in certain sites and their concentration falls very rapidly after birth.

The glyceryl ethers have also been identified in meconium from human sources.

It is considered that their formation is associated with cells of mesenchymal type, probably early forms of multilocular fat cells.

The literature has been reviewed and their presence in adult bone marrow, erythrocytes and spleen can thus be explained. The presence of glyceryl ethers in atheromatous plaques is associated with other fractions of unsaponifiable material such as pigments, carotenoids and cholesterols, and is probably not specific.

It has been shown that their experimental use in animals, and their clinical use in man is to initiate the first stage in wound healing if, and only if, this is pathologically inhibited.

Therapeutic effect in the treatment of non-healing wounds and radiation injuries has been outlined, and the suggestion is made that their action is upon tissues of mesenchymal origin.

Experiments have been put in hand to show if these substances have any prophylactic effect against the action of ionising radiation in minimal doses.

No toxic effects have been noticed in prolonged oral administration in man, although individual tolerance in terms of nausea vary considerably.

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EGER[79] (*see* p. 268) has since published his results: *Strahlentherapie, 105* (1958) 296.

We would suggest for consideration that this irradiation dose, leading to stimulation of repair, accelerates activity of mucosal cells of the upper intestine, where we have suggested that the AGE are metabolised. Allyl alcohol is a raw product from which batyl alcohol may be synthesised[23].

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