The liver glycerides contained considerably less hexadecenoic acids, somewhat less oleic and polyethenoid C_{18} acids, and considerably more polyethenoid C_{20} and C_{22} acids; the degree of unsaturation of the unsaturated components was similar in both liver and blubber glycerides. No comparable data are available for the liver and blubber glycerides of the whale or any other marine mammal, whilst similar comparisons in a number of fish species seem to indicate that no generalization can yet be made. Thus, in the sturgeon (Lovern, 1932*b*) and groper (Shorland & Hilditch, 1938) there is less fat in the liver than in the peritoneal cavity and pancreas (sturgeon) or the head (groper); but the component acids of liver and depot fats are similar in composition in the respective fish. This also obtains in the tunny (Lovern, 1936), in which the liver and flesh contain about the same proportion of fat. On the other hand, in halibut and turbot (Lovern, 1932*a*, 1937) the liver is the main fat depot, and the flesh contains but little fat: in both of these instances the unsaturated C_{16} acid contents of the liver glycerides are much higher, and the amount of polyethenoid C_{20} and C_{22} acids much lower, than in the corresponding flesh fats. This lack of correlation stands in contrast to the fats of land animals in which (Hilditch & Shorland, 1937) the liver glycerides are distinguished by definitely lower contents of stearic acid, and higher contents of hexadecenoic and of polyethenoid acids of the C_{20} and C_{22} series, in comparison with the corresponding depot fats.

SUMMARY

1. The composition of the blubber and liver lipids from two specimens of the common seal (*Phoca vitulina* L.) has been investigated.

2. The component acids of the blubber glycerides of the two animals were not dissimilar, but differed considerably from that of the grey seal (*Halichoerus grypus*). These differences are probably due partly to difference in species, but it also appears likely that differences in the food ingested by individual seals may be responsible to some extent for variations in the fat laid down.

3. The liver lipids of seals also appear to differ in amount and in their composition. One of the two common seal livers examined was exceptionally rich in glycerides as compared with phosphatides. Contrary to what was observed in the grey seal, the liver glycerides of the common seal are very similar in composition to its blubber oil.

4. So far, no correlation has been discernible between the composition of the liver glycerides and the glycerides of the flesh, head or other tissues of marine animals which may function as fat depots.

It is a pleasure once more to offer our thanks to Prof. A. N. Worden and to Dr L. Harrison Matthews for again providing us with interesting experimental material of authentic origin.

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The Action of Trypsin on Insulin

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In previous studies (Butler, Dodds, Phillips & Stephen, 1948) it was found that the action of trypsin on insulin is comparatively slight. It appeared likely that the slow action observed was due to a small proportion of chymotrypsin (about 0·5%) known to be present in the trypsin, and it was doubtful if there was any residual effect which might be ascribed to trypsin itself. In some further experiments with the same materials it was found that the extent of the action was rather variable, and appeared to depend on several factors, e.g. the method of preparing the insulin solution, the age of the solution at the time of enzyme addition. The possibility that trypsin might cause a slight hydrolysis of insulin without decreasing its potency has led us to re-examine its action, using eventually

a purified trypsin kindly given us by Drs J. H. Northrop and M. Kunitz, and a sample of insulin which had been several times recrystallized.

EXPERIMENTAL AND RESULTS

The effect of trypsin on insulin solutions prepared in several ways

These experiments were carried out as described in a previous paper (Butler *et al.* 1948), using the same trypsin, and an insulin concentration of 5 mg./ml. The insulin solutions were made up as indicated in Table 1. The extent of hydrolysis certainly varied with the method of preparation of the solution, but in most cases was comparatively small in the first 24 hr., and only became considerable after that period. This was probably due to the slow action of the chymotrypsin present on the insulin, which converts the latter into products upon which trypsin can act. Initial treatment with alkali, apparently, makes the insulin more susceptible to attack under these conditions.

Action of purified trypsin

The action of the purified enzyme on the commercial insulin was found, as might be expected, to be appreciably less especially in long periods than that of the trypsin used above, but a real residual action still remained. Table 2 shows the results obtained in two distinct experiments. In both cases there is an increase of non-protein nitrogen (N.P.N.) amounting to about 7% in 1 day and 12% in 2–3 days, and the amino N increases at the same time to about 10%, which would signify a quite appreciable splitting of about 6 peptide bonds/insulin unit of 12,000. At the end of the experiments, the pH of the solutions was adjusted to 5·4 and the precipitates which formed were filtered off and dried. Their insulin potency, kindly determined for us by Boots Pure Drug Co. Ltd., was 56–59% of that of the original insulin. It is evident that in the long-continued action of trypsin there had been considerable loss of potency.

Action of purified trypsin on recrystallized insulin

There remains some doubt whether the non-protein N formed comes from insulin or extraneous substances. The insulin used was Boots zinc insulin, potency 22·8 units/mg. This was found to give a single sharp peak in the electrophoretic diagram. It was recrystallized four times, the first three by the method of Romans, Scott & Fisher (1940), and the last time by dissolving in dilute acetic acid and adding NaOH to pH 4·5. It should be pointed out that, according to a recent paper by Lens (1948), this procedure may result in the formation of some denatured material. The results obtained by digesting this insulin (5 mg./ml.) with 135×10^{-4} units of the purified trypsin in 100 ml. are shown in Table 3 A. A similar control experiment without any trypsin present is shown in Table 3 B. It is evident that the change in N.P.N. in the presence of trypsin was not much greater than that in a similar buffer containing no trypsin. The insulin, however, lost a rather greater amount of potency in the presence of the trypsin than in its absence. The potency of the recovered material from the trypsin solution after 72 hr. was greater than that after 48 hr.; this may be connected with the fact that a smaller proportion of the insulin was actually

Table 1. *Tryptic action on insulin solutions prepared in various ways*

(Non-protein N (N.P.N.) expressed as % of total N.)

A		*B*		*C*		*D*		*E*	
Time (hr.)	N.P.N (%)	Time (hr.)	N.P.N. (%)	Time (hr.)	N.P.N. (%)	Time (hr.)	N.P.N. (%)	Time (hr.)	N.P.N. (%)
0	—	0	7·6	0	7·3	0	9·7	0	11·6
17·5	9·7	23	14·7	18	10·9	23	13·6	18	17·5
44·5	19·2	46	18·0	42	44·7	48	45·6	42	23·2

A. Insulin mixed with phosphate buffer, pH 7·6, before adding enough alkali to bring it into solution. Trypsin added at once.
B. Same as *A*, but trypsin added 70 hr. after making up solution.
C. Insulin dissolved in 0·02 N-NaOH and same quantity of phosphate buffer then added. Trypsin added at once.
D. Same as *C*, but trypsin added 21 hr. after making up solution.
E. Same as *A*.

Table 2. *Action of purified trypsin on insulin*

(Digest contains 5 mg. insulin/ml. in M/24 phosphate buffer, pH 8·2.)

Trypsin: 44×10^{-4} units in 30 ml.			Trypsin: 72×10^{-4} units in 50 ml.		
Time (hr.)	N.P.N. (%)	Amino N (%)	Time (hr.)	N.P.N. (%)	Amino N (%)
0	5·5	4·4	0	6·6	4·4
3	5·7	—	23	14·4	9·3
21	12·5	—	47	18·9	9·5
44·5	12·5	—			
68·5	17·2	10·0			
N precipitated at pH 5·4, 82%			N precipitated at pH 5·4, 84·5%		
Potency of precipitate 13·4 units/mg.			Potency of precipitate 12·7 units/mg.		

Table 3. *Comparison of changes in digest with those in buffer alone*

A. Action of purified trypsin on 4 times recrystallized insulin (25°)

Time (hr.)	0	48	72
N (% of T.N.)* in trichloroacetic filtrate. (N.P.N.)	4·0	7·6	11·1
N (% of T.N.) in isoelectric filtrate	—	6·4	11·5
Amino N (% of T.N.) in recovered insulin	4·4	6·3	5·8
Amide N (% of T.N.) in recovered insulin	9·9	8·4	9·0
Mean potency of precipitate (units/mg.)	22·96	15·6	18·7
Limits of error, $P=0·95$	21·01–25·09	13·69–17·71	15·89–22·13

B. Change of insulin with time in buffer solution, pH 8·4 (25°)

Time (hr.)	0	24	48	72
(*a*) N (% of T.N.) in trichloroacetic filtrate. (N.P.N.)	4·9	6·2	8·6	10·0
Potency of precipitate (units/mg.)	22·9	—	—	20·6
Limits of error, $P=0·95$	—	—	—	18·5–22·9
(*b*) N (% of T.N.) in isoelectric filtrate	0·0	2·2	3·2	4·8
Amide N (% of T.N.) in recovered insulin	9·3	9·4	9·5	8·3

* T.N. = Total nitrogen.

recovered in the former case. There was a slight increase in the percentage of amino N in the recovered insulin from trypsin, which may indicate the breakage of one or two peptide bonds. The results shown in lines (*a*) and (*b*), Table 3B, were obtained in separate experiments. The N.P.N. values obtained in this experiment seem rather high; they are quoted because the potency of the insulin recovered from the buffer after 72 hr. was also determined. A redetermination of the N.P.N. in the trichloroacetic acid filtrate after 72 hr. (exp. *b*) gave a value only about 3% greater than the N in the isoelectric filtrate. It is evidently difficult to reproduce the N.P.N. formed in buffer solutions after long periods of time.

Dr K. O. Pedersen of Uppsala has kindly examined in the ultracentrifuge the products of trypsin action dissolved in a buffer solution containing Na_2HPO_4 (0·10 M) and NaH_2PO_4 (0·05 M). The sedimentation constant of the bulk of the material was found to be the same as that of untreated insulin (Pedersen, 1948), but a spreading of the sedimentation curves indicated that the material had become less homogeneous. The possibility that the degradation of insulin in the buffer solution was due to the contamination of the insulin by a proteolytic enzyme was considered. No evidence of the existence of the latter could be obtained when insulin was added to the haemoglobin substrates suggested for the assay of trypsin and pepsin by Anson (1938), but the concentration necessary to produce the effect with insulin could be too small to detect by the latter method.

Since trypsin exerts an amidase activity on certain amides (Bergmann, Fruton & Pollock, 1939; Schwert, Neurath, Kaufman & Snoke, 1948), and since insulin contains 8·9% of its nitrogen as amide N (Chibnall, 1942), we have also attempted to find if any of the latter is liberated by trypsin. The amide N was determined in a conventional manner (see Sanger, 1945), but no significant diminution was observed in trypsin-treated insulin.

Purification of insulin by treatment with trypsin

If, unlike other proteins, insulin is resistant to trypsin it should be possible to purify it to some extent, at least, by tryptic digestion. A crude insulin preparation, kindly given to us by Boots Pure Drug Co. Ltd., had a potency of 8·2 units/mg. This material (0·5 g.) was dissolved in 85 ml. of phosphate buffer, pH 7·8, and treated with crystalline trypsin (500×10^{-4} units) for 19 hr. at 25°. The pH was then adjusted to 5·2 with 0·1 N-HCl and the precipitate filtered off (dry wt. 59 mg.). Its potency, as determined by Boots, was 17·0 units/mg., i.e. the potency of the insulin had been doubled, but there was a considerable loss, since only 1000 units were recovered from an initial 4000. It is possible that purified trypsin would give a more satisfactory recovery, and that trypsin action may be a useful step in the purification of trypsin-resistant proteins.

DISCUSSION

Every increase in the purity of the enzyme preparation and of the insulin has resulted in a decrease of the action observed, and under the best conditions the change produced is slight. The increase in non-protein nitrogen in the presence of trypsin is of the same order as that in the buffer alone. There is, however, a slight increase in the free amino nitrogen of the recovered insulin and the potency of the recovered material is certainly appreciably less. It is therefore possible that the trypsin breaks one or two bonds in the insulin molecule, per unit of mol. wt. 12,000, without any non-protein peptide being liberated. The ultracentrifuge also shows a change in the nature of the recovered insulin. Although its mean sedimentation constant is unchanged, there is a greater spread of sedimentation rate, indicating an increased heterogeneity of the material. This might be due to a slight denaturation affecting the shape of the molecules and the way in which the submolecules aggregate.

SUMMARY

1. The action of trypsin on insulin has been reexamined. Purified trypsin has less action than the trypsin originally used.

2. Using four times recrystallized insulin and purified trypsin, the non-protein nitrogen formed is not much greater than that produced in the buffer

alone, but in the presence of trypsin the physiological potency is diminished to a somewhat greater extent than in its absence. Ultracentrifugal examination of the recovered insulin shows it to have become less homogeneous than the original.

3. The action of trypsin on insulin is thus slight, but the possibility of the hydrolysis of one or two peptide bonds, with a diminution of potency of 10–20%, is not excluded.

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Studies in the Biochemistry of Micro-organisms

80. THE COLOURING MATTERS OF *PENICILLIUM ISLANDICUM* SOPP. PART 1. 1:4:5-TRIHYDROXY-2-METHYLANTHRAQUINONE

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Penicillium islandicum Sopp is a species included by Thom (1930) in the Funiculose series of the division of the Penicillia known as the Biverticillata-symmetrica. The species was first described and named by Sopp (1912). He isolated it in Norway from a mouldy specimen of skyr received from Reykjavik, Iceland. Skyr* is a bacterially soured milk peculiar to Iceland, and is somewhat similar in nature to Bulgarian yogurt. A number of other strains have been isolated subsequently in different parts of the world. Colonies of *P. islandicum* are characterized by their three-coloured appearance, 'a green conidial zone near the margin and progressively overgrown with red orange to red hyphae in the central areas, reverse at first orange to sordid yellow orange shades, later becoming rich red shades' (Thom, 1930). We are engaged in a study of this complex mixture of colouring matters, and it is the purpose of the present communication to describe the isolation of one of them and its identification as the hitherto undescribed 1:4:5-*trihydroxy-2-methylanthraquinone*.

* Thom (1930, p. 466), in a description of *P. islandicum* Sopp, says 'species found on the Island of Skyr'. Sopp's (1912, p. 162) original description states 'Der Pilz wurde auf isländischem Skyr gefunden'.

The new colouring matter, which was isolated by solvent extraction from five different strains of *P. islandicum* grown on Czapek-Dox 5% glucose solution, was obtained in yields of 3% of the dried mycelium of strain 1036. The total yield of the fat-free complex mixture of colouring matters was rather remarkable. It amounted to 20% of the dried mycelium. The nature of the other colouring matters is being investigated.

The new colouring matter forms large, dark red, lustrous plates or leaflets, m.p. 218°. It has the molecular formula $C_{15}H_{10}O_5$, contains one methyl group attached to carbon, no methoxyl group, and forms a triacetate, m.p. 208°, and a trimethyl ether, m.p. 161°, mol. wt. 314, 320. It is not soluble in aqueous sodium carbonate, but dissolves readily in sodium hydroxide giving a deep violet solution. Its solution in cold concentrated sulphuric acid is bright purple red in colour with a fiery red fluorescence. Its general behaviour is that of a polyhydroxyanthraquinone.

Its molecular structure as 1:4:5-trihydroxy-2-methylanthraquinone was established as follows. Since its molecular formula is $C_{15}H_{10}O_5$, and it contains one methyl group and three hydroxyl groups (formation of a triacetate and a trimethyl ether), it