detected. In both cases malonic acid was by far the most abundant product. The traces of glutaric and adipic acid in the oxidation products of the C_{18} and C_{19} acid respectively, can be explained by the presence of C_{20} and C_{21} pentaenoic acid, from which the above dicarboxylic acids are the known oxidation products. However, the possible presence of small amounts of an isomeric octadecatetraenoic acid cannot be excluded.

The main component of the isolated material therefore has the structure:

So far all the other highly unsaturated acids isolated from pilchard oil which have been reported in this series, as well as all the polyethenoid acids of marine and mammalian origin isolated by Klenk and his co-workers (see Klenk, 1957), exhibit methylene-interrupted conjugation.

SUMMARY

1. *cis-n*-Octadeca-6:9:12:15-tetraenoic acid has been isolated from South African pilchard oil, and some of its properties have been determined.

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W. Albrecht and X-ray long spacings by Dr F. H. Herbstein. The melting point of the octadecatetraenoic acid was determined by Dr S. C. Mossop. This paper is published with the permission of the South African Council for Scientific and Industrial Research.

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Changes in the Distribution of Phosphorus in Platelet-Rich Plasma During Clotting

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In the course of work which has suggested that adenosine triphosphate is concerned in the accumulation of biologically active amines by platelets (Born, Ingram & Stacey, 1956; Born & Hornykiewicz, 1957; Born & Gillson, 1957) it was observed that, when platelet-rich plasma clots, adenosine triphosphate rapidly disappears from the platelets. This paper reports these experiments and others made to determine what happens during clotting to the phosphate groups of adenosine triphosphate and to other phosphate compounds which are present in plasma and in platelets. Some of the results have been published in preliminary form (Born, 1956a, b, 1957).

METHODS

All glassware used in the isolation of rabbit and human platelets was coated with silicone MS 1107 (Hopkin and Williams Ltd.). When pig platelets were used this was not always done, because pig platelets have little tendency to clump even when in contact with glass.

Blood samples. Pig blood was obtained in the slaughter house. Immediately after the pig was electrically stunned, the throat was slit and venous blood was collected in a polythene container immersed in ice. Rabbit blood was obtained by cardiac puncture. Human blood was obtained by venepuncture. Blood samples were mixed with sodium citrate (final concn. 13 mM) to prevent clotting.

Platelets were isolated at 0–2°. Blood was centrifuged at 500 g for 15–20 min. and supernatant plasma was

collected. This was called platelet-rich plasma and a sample of it was used for counting platelets. When plasma had to be freed from platelets, small amounts were centrifuged at 25 000 *g* for 5 min. and large amounts at 6000 *g* for 20 min. Most platelet sediments were almost free of erythrocytes and leucocytes (less than one leucocyte/10⁴ platelets). To clot plasma it was incubated at 37° in glass centrifuge tubes, which were not treated with silicone, and CaCl₂ was added to give a concn. of 10–25 mM. To separate a clot from the serum, the clot was squeezed to the bottom of the centrifuge tube with a glass rod, and the tube was then centrifuged as described for sedimenting platelets. Very little serum remained in the clot.

Samples of plasma with and without platelets, separated platelets, serum and clot, were extracted first with aqueous trichloroacetic acid for acid-soluble compounds of phosphorus and then with chloroform-methanol for lipid phosphorus; the residue which remained was analysed for total phosphorus.

Determination of phosphorus. Phosphorus was determined in all fractions by the method of Berenblum & Chain (1938). The details of the procedure were as follows:

Acid-soluble phosphorus was extracted at 0–2°. Ice-cold trichloroacetic acid was added to the sample to a final concn. of 10% (w/v). The sample was dispersed in a glass homogenizer and centrifuged at 3000 *g* for 5 min. The clear supernatant was transferred to another test tube. The precipitate was dispersed in more acid, centrifuged again and the supernatant solution added to the first. The solution was extracted four times with an equal volume of ice-cold ether to remove the trichloroacetic acid. The ether was removed by bubbling nitrogen through the solution, which was then frozen at –10° until samples were analysed for inorganic phosphorus, 10 min. phosphorus, adenosine triphosphate (ATP) and total acid-soluble phosphorus, as follows:

Inorganic phosphorus was estimated by adding a sample directly to the acid molybdate reagent of Berenblum & Chain.

10 min. phosphorus was the phosphorus set free by hydrolysis in *N*-HCl at 100° in 10 min.

ATP was determined by the firefly-luminescence reaction (Strehler & Totter, 1954). Extracts of plasma and serum inhibited the luminescence; therefore internal standards were used when ATP in plasma and serum was determined.

Total acid-soluble phosphorus was determined in a sample which had been digested in a micro-Kjeldahl flask for 30 min.–1 hr. with 0.25 ml. of conc. H₂SO₄, 2 ml. of water and 2 ml. of H₂O₂ (30%, w/v).

Lipid phosphorus was extracted from the trichloroacetic acid precipitate. The precipitate was washed twice with water and dried in a vacuum over P₂O₅. It was then extracted twice with chloroform-methanol (2:1, v/v) according to Folch, Ascoli, Lees, Meath & LeBaron (1951). Samples of the extract were evaporated almost to dryness, digested (see above) and analysed for phosphorus, giving lipid phosphorus.

The residue which was left after acid-soluble phosphorus and lipid phosphorus had been extracted was resuspended in water. Samples of the suspension were digested and analysed for phosphorus to give residue phosphorus.

Protein nitrogen. This was determined by the micro-Kjeldahl method.

5-Hydroxytryptamine. This was extracted from platelets as described by Hardisty & Stacey (1955) and determined by assay on the isolated rat uterus, in oestrus, in a 2 ml. bath.

RESULTS

Concentrations of adenosine triphosphate in platelets

Platelets were found to contain ATP in high concentrations. Results are set out in Table 1, which includes figures, obtained by the same and by other methods, for the concentrations of ATP in other tissues to permit comparisons to be made.

Table 1 shows that platelets contain a higher concentration of ATP than all other tissues so far examined, except adrenal medulla and mammalian striated muscle. The concentration is about equal to that in such muscle, and it is higher than that found in the mitochondria of heart muscle and of liver.

Distribution of phosphorus in platelets

The distribution of phosphorus in platelets is shown in Table 2. The platelets were extracted first with aqueous trichloroacetic acid and then with chloroform-methanol as described under Methods, and extracts and residue were analysed for phosphorus.

In human platelets, ATP phosphorus represented about 56% of total phosphorus soluble in acid. In one experiment with pig platelets the percentage was as high as 78%. Acid-soluble phosphorus represented about one-half and lipid phosphorus about one-third, of the total phosphorus in the platelets. There were 5–6 × 10¹⁰ pig platelets/g. (moist wt.) (see also Fantl & Ward, 1956), and the dry weight was one-fifth of the moist weight. On the assumption that phosphorus makes up 4% of the weight of lipids extracted by chloroform-methanol, dry platelets contained 6–7% of phospholipid; Erickson, Williams, Avrin & Lee (1939) found 12% in human platelets.

Breakdown of adenosine triphosphate during clotting

When blood clots, platelets release 5-hydroxytryptamine into the serum (Rand & Reid, 1951; Zucker & Borrelli, 1955). This release does not depend on the formation of the fibrin clot (Hardisty & Pinniger, 1956). In view of the possibility that in platelets 5-hydroxytryptamine is associated with ATP (Born *et al.* 1956; Born & Gillson, 1957) it was of interest to determine whether ATP was also released from platelets during clotting.

Citrated human plasma was divided into two portions. The platelets were removed from one by centrifuging it at 25 000 *g* for 20 min. at 0–1°. Calcium was added to both and they were incubated at 37° for 1 hr. The clots were separated from

the sera and the amounts of 5-hydroxytryptamine and ATP were determined in platelets, clots and sera.

Table 3 shows the results. Before clotting, the platelets contained 24.4 $\mu\text{m-moles}$ of 5-hydroxytryptamine; after clotting, 14.1 $\mu\text{m-moles}$ of 5-hydroxytryptamine, or 57 %, were found in the

serum. Of the 370 $\mu\text{m-moles}$ of ATP originally present in the platelets, only 51 $\mu\text{m-moles}$, or 14 %, were found in the clot which contained the platelets or what remained of them, and none was found in the serum. This suggested that platelet ATP breaks down during clotting.

Table 1. *Concentration of adenosine triphosphate in platelets compared with its concentration in other tissues*

Species	Tissue	Concn. of ATP		No. of determinations	Reference
		Mean and range ($\mu\text{moles/g. moist wt.}$)	S.E.M.		
Rabbit	Platelets	5.4 (4.2 and 6.5)	—	2	This paper
Pig	Platelets	4.3 (2.8–5.7)	—	5	This paper
Man	Platelets	3.8 (2.9–4.5)	—	5	This paper
Man	Erythrocytes	1.2	0.05	15	Gourley (1952)
Rat	Heart	1.7	0.03	6	Parker (1954)
Rat	Brain	1.8	0.13	6	Parker (1954)
Rat	Skeletal muscle	4.5	0.33	11	Parker (1954)
Frog	Skeletal muscle	2.2	—	38	Fleckenstein, Janke, Davies & Krebs (1954)
Cow	Adrenal medulla	8.1 (6.5 and 9.8)	—	2	Blaschko, Born, D'Iorio & Eade (1956)
		($\mu\text{moles/g. of protein N}$)			
Rabbit	Platelets	288	51	6	This paper
Pig	Platelets	266	14	5	This paper
Man	Platelets	225	23	7	This paper
Rat	Heart-muscle sarcosomes	138	—	3	Slater & Holton (1953)
Mouse	Liver mitochondria	143	—	1	Kielley & Kielley (1951)
Cow	Adrenal-medullary granules (bottom fraction)	1720	59	10	Blaschko <i>et al.</i> (1956)

Table 2. *Distribution of phosphorus in platelets*

Figures are in $\mu\text{mg. atoms of P}/10^8$ platelets. (1) indicates that the platelets were obtained from plasma which had been dialysed against phosphate-free solution; (2) represents the mean result of 17 other determinations which are not shown in this table; (3) the figure is calculated on the assumption that the Fantl & Ward (1956) value for 7 min. P represented two-thirds of the ATP P originally present in the platelets.

	Acid-soluble P			Lipid P	Residue P	Total
	Inorganic P	Total P of ATP	Total acid-soluble P			
Pig platelets	4.8	41.4	53.3	33.6	9.3	96.2
Pig platelets (1)	4.4	17.4	30.0	25.1	4.7	59.8
Pig platelets (1)	—	33.8	52.6	33.9	4.0	90.5
Pig platelets (1)	5.5	20.5	58.0	25.6	6.2	89.8
Mean	—	28.3	48.5	29.6	6.0	84.1
Human platelets	—	—	44.5	44.3	—	—
Human platelets	—	—	29.7	41.7	—	—
Human platelets	—	—	32.3	33.9	7.1	73.3
Human platelets (1)	4.5	9.5	21.0	30.0	5.2	56.2
Mean	—	18 (2)	31.9	37.5	—	—
Human platelets (Fantl & Ward, 1956)	6.6	28 (3)	57	—	—	—

Normal platelets contain much more ATP than adenosine diphosphate (ADP). The value for 10 min. phosphorus in trichloroacetic acid extracts of platelets measures mostly ATP. In the experiment referred to, the amount of ATP which had broken down was $0.32 \mu\text{mole}$, and the amount of 10 min. phosphorus which had disappeared was $0.51 \mu\text{g. atom}$. This suggested that ATP had lost more than the terminal phosphorus atom.

The next series of four experiments was made to determine how rapidly ATP disappeared from the platelets after clotting was induced. One of these experiments was as follows:

Human plasma (2.5 ml.) containing 4.12×10^8 platelets/ml. was incubated in a water bath at 37° with 0.06 ml. of m-CaCl_2 to start the clotting process. The sample was immediately cooled in ice and centrifuged at $25\,000 g$ for 5 min. at $0-1^\circ$. Other 2.5 ml. samples were incubated with CaCl_2 in the same way. Clotting became visible after about 2 min. Different samples were rapidly cooled and centrifuged after 2.5, 5, 10, 20 and 30 min. The sediments, consisting of clot and platelet material, were extracted with trichloroacetic acid as already described. Two control samples were incubated at 37° without added CaCl_2 . They did not clot. After 20 and 30 min. they were centrifuged and the sediments of platelets were extracted.

In unclotted plasma the concentrations of ATP, 10 min. phosphorus and inorganic phosphorus in the platelets remained practically constant. In

clotting plasma, on the other hand, during the first 10 min. there was a rapid decrease in the concentration of ATP in the platelets. After that the rate of disappearance of ATP slowed down. This is shown in Fig. 1, which also shows that all the 5-hydroxytryptamine originally present in the platelets was

Fig. 1. Disappearance of ATP in platelets which are present in plasma during clotting, and the release of 5-hydroxytryptamine (HT) from them. The experiment is described in the text. Abscissa: time (min.) after addition of CaCl_2 (final concn. 0.025 M). ●, ATP (as percentage of that originally present in the platelets); O, HT appearing in the serum (as percentage of that originally present in the platelets).

Table 3. Breakdown of adenosine triphosphate and release of 5-hydroxytryptamine (HT) when platelets are present during clotting

Figures refer to 11.5 ml. of human plasma, which contained 5×10^8 platelets/ml.

Conditions	Source	ATP ($\mu\text{m-moles}$)	HT ($\mu\text{m-moles}$)
Clotting in the absence of platelets	Platelets (removed before clotting)	370	24.4
	Clot	About 10	0.6
	Serum	Nil	0.6
		380	25.6
Clotting in the presence of platelets	Clot	51	11.8
	Serum	Nil	14.1
		51	25.9

Table 4. Changes in adenosine triphosphate, 10 min. phosphorus, and inorganic phosphorus extracted from the clot when platelet-rich plasma clots

Time after adding Ca (min.)	Decrease in ATP ($\mu\text{m-moles}$)	Decrease in 10 min. P ($\mu\text{mg. atoms}$)	Increase in inorganic P ($\mu\text{mg. atoms}$)	Decrease in 10 min. P not accounted for as increase in inorganic P ($\mu\text{mg. atoms}$)	Ratio (10 min. P unaccounted for) decrease in ATP
5	27	137	84	53	2.0
10	31	157	97	58	1.9
20	34	192	158	34	1.0
30	36	211	197	14	0.4

released into the serum during the time in which ATP disappeared most rapidly from the platelets.

Table 4 shows the changes that occurred in ATP, 10 min. phosphorus, and inorganic phosphorus. The decrease in the concentration of ATP was accompanied by a disappearance of 10 min. phosphorus, only part of which was accounted for by an increase of inorganic phosphorus. The difference, which up to the tenth minute represented almost exactly 2 moles of phosphate for each mole of ATP broken down, disappeared from the trichloroacetic acid extracts. This suggested the possibility that during clotting phosphorus was transferred from ATP in the platelets to another component of the platelets or to the plasma. After 20 and 30 min. an increasing proportion of the 10 min. phosphorus which disappeared could be accounted for as inorganic phosphorus. After 30 min. the increase in inorganic phosphorus was almost as much as the decrease in 10 min. phosphorus.

Table 5 shows that inorganic phosphorus which was associated with the clot also increased when plasma clotted in the absence of platelets. The source of this inorganic phosphorus was presumably the inorganic phosphorus present in the plasma.

Redistribution of inorganic phosphorus and 10 min. phosphorus between platelets and plasma during clotting

The next step was to find out whether the 10 min. phosphorus which could not be found in trichloroacetic acid extracts of a clot appeared in the acid extract of the serum from which the clot had been separated. In the serum it might appear either as 10 min. phosphorus or as inorganic phosphorus.

In order to be able to determine the small changes in inorganic phosphorus and 10 min.

phosphorus which clotting might be expected to bring about in this way it was necessary, before inducing clotting, to reduce the comparatively high concentration of inorganic phosphorus (about 1 $\mu\text{g. atom/ml.}$) which is present in normal plasma. This was done by dialysing plasma against large volumes of Krebs-Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1951), which was modified so as to contain no phosphate, before the plasma was made to clot. One experiment of this kind was as follows.

A sample of citrated human plasma (5 ml.), which contained 6×10^8 platelets/ml., was centrifuged to sediment the platelets. Platelets and plasma were extracted with trichloroacetic acid. Another sample of the same plasma (11.1 ml.) was dialysed for 21 hr. at 2–4° against 2 l. of a solution having the following composition (mm): NaCl, 119; KCl, 4.8; CaCl_2 , 2.6; MgSO_4 , 1.2; NaHCO_3 , 25; dextrose, 1.12; ethylenediaminetetra-acetate, 3.6; $\text{O}_2 + \text{CO}_2$ (95:5) was bubbled through the solution, which had a pH of 7.5.

After dialysis the volume of the plasma was 11.4 ml. The changes which dialysis brought about in the concentrations of inorganic phosphorus and 10 min. phosphorus in the plasma are shown in Table 6. The inorganic phosphorus was reduced to about one-twentieth of its original concentration, and material that behaved like 10 min. phosphorus in the plasma was reduced to about one-tenth. In the platelets variable amounts of 10 min. phosphorus disappeared while the amount of inorganic phosphorus in them increased slightly. After dialysis, there was about four times more 10 min. phosphorus in the platelets than in the plasma.

A volume (5 ml.) of dialysed plasma was centrifuged. The sedimented platelets and the supernatant plasma were extracted with trichloroacetic acid. Dialysed plasma (6.3 ml.) was incubated at 37° with CaCl_2 for 30 min. The clot, which contained the platelets or their remnants, was separated from the serum and both were extracted with trichloroacetic acid. These extracts were analysed for inorganic phosphorus, 10 min. phosphorus, and ATP. A similar dialysis experiment was made with pig plasma containing 4.1×10^8 platelets/ml.

Table 5. Increase in the amount of inorganic phosphorus in the clot when platelet-free plasma clots

Time after adding Ca (min.)	Inorganic P ($\mu\text{mg. atoms/ml.}$ of plasma)
0	51
20	76
30	118

Table 6. Concentrations of phosphorus ($\mu\text{mg. atoms/ml.}$) in platelet-rich plasma before and after dialysis against a solution free of phosphate

	Human plasma			Pig plasma		
	Before dialysis	After dialysis	Change	Before dialysis	After dialysis	Change
Inorganic P in plasma	452	20	–432	1910	101	–1809
10 min. P in plasma	204	29	–175	374	32	–342
10 min. P in platelets	207	112	–95	132	122	–10
Inorganic P in platelets	35	36	+1	25	39	+14

Table 7. *Changes in the concentrations of inorganic phosphorus, 10 min. phosphorus, and adenosine triphosphate in plasma and in platelets during clotting*

Changes in concentration are expressed in $\mu\text{mg. atoms/ml.}$ of plasma for plasma constituents and in $\mu\text{m-moles/platelet}$ content of 1 ml. of plasma for platelet constituents.

	Human plasma	Pig plasma
Decrease in 10 min. P in platelets	82.2	86.8
Increase in 10 min. P in plasma	20.3	14.8
Increase in inorganic P in platelets	6.1	Nil
Increase in inorganic P in plasma	35.8	35.5
10 min. P unaccounted for	20.0	36.5
Decrease in ATP in platelets	17.0	18.2
Ratio $\left(\frac{10 \text{ min. P unaccounted for}}{\text{decrease in ATP in platelets}} \right)$	1.2	2.0

The results of these two experiments are shown in Table 7. In both experiments most of the 10 min. phosphorus disappeared from the platelets, but only a quarter or less was found in the serum. The concentration of inorganic phosphorus increased slightly in the platelets and considerably in the serum, and these increases accounted for a half or less of the 10 min. phosphorus lost by the platelets. Therefore a quarter to a third of the 10 min. phosphorus of the platelets remained unaccounted for. In one of the experiments $1.2 \mu\text{mg. atoms}$, and in the other exactly $2 \mu\text{mg. atoms}$, of 10 min. phosphorus disappeared for each $\mu\text{m-mole}$ of ATP which disappeared from the platelets. This again suggested that the disappearance of 10 min. phosphorus from the trichloroacetic acid extracts indicated the transfer of more than one but not more than two phosphate groups of platelet ATP to an unknown receptor substance.

Redistribution of phosphate in plasma clotting with and without platelets

To discover where the 10 min. phosphorus which disappeared from platelets was going, 30 ml. of citrated human plasma, containing 4.2×10^8 platelets/ml., was dialysed for 24 hr. against the phosphate-free solution. Plasma (15 ml.) was then centrifuged to remove the platelets and the sedimented platelets were extracted with trichloroacetic acid, water and chloroform-methanol as described under Methods. Platelet-free plasma was made to clot with CaCl_2 in one series of tubes and platelet-rich plasma in another, each tube containing 2.5 ml. of plasma. After 0, 4, 8, 16 and 32 min. the contents of one tube of each series were mixed with trichloroacetic acid and extracted in the same way as the platelets.

Fig. 2 shows the changes in the amounts of phosphorus present in the different fractions. The most striking change was the increase in the amount of phosphorus found in the residue; this occurred only when platelets were present during clotting but not when they were absent. The in-

Fig. 2. Changes in the concentrations of phosphorus and nitrogen during clotting of plasma. The experiment is described in the text. Plasma free of platelets: ○, P in residue. Plasma rich in platelets: ●, P, in residue; ○, N in residue; ●, 10 min. P; ●, P extracted by chloroform-methanol.

crease was maximal after 8–16 min. After 32 min. the residue had lost most of the extra phosphorus. The amount of nitrogen in the residue increased and decreased concurrently with the phosphorus.

The concentration of 10 min. phosphorus decreased before and during the increase of phosphorus in the residue. The amount of lipid phosphorus also decreased, and together these decreases were almost equal to the increase in residue phos-

phorus. There was a small increase in inorganic phosphorus which may have come from 10 min. phosphorus, although other sources were not excluded. None of these changes was found in the plasma which had clotted in the absence of platelets. It seemed therefore that when platelets were present in plasma during clotting, some acid-labile (10 min.) phosphorus and part of the lipid phosphorus became associated with the residue in such a way that they could no longer be extracted by trichloroacetic acid or by chloroform-methanol.

The total number of experiments made to investigate these changes was nine. In the first three, pig plasma was used and clotting was allowed to go on at 37° for 1 hr. before trichloroacetic acid was added. In two of these experiments the changes were qualitatively similar to those which have just been described; in the third the changes were so small as to be within the error of the methods used to extract phosphorus and to determine it.

In six experiments human plasma was used. In three, the changes were all similar to those which have been described. In the other three, the decreases of 10 min. phosphorus and of lipid phosphorus were also found, but no determinations were made of changes of phosphorus in the residue. The magnitude of the changes observed varied from one experiment to another.

Evidence that the decrease in extractable lipid phosphorus occurred mostly in the plasma

The question arose whether the decrease in extractable lipid phosphorus occurred in the platelets or in the plasma, or in both. The procedure used to answer this question is exemplified by the following experiment.

Pig plasma (120 ml.) containing 4.7×10^8 platelets/ml. was dialysed for 20 hr. against phosphate-free Krebs-Ringer bicarbonate solution. After dialysis the volume of the plasma was 128 ml. A sample was analysed for total phosphorus.

A volume (50 ml.) of dialysed plasma was centrifuged and the sediment of platelets extracted. The supernatant plasma, poor in platelets, and 50 ml. of dialysed plasma rich in platelets were incubated and made to clot. After 30 min. the clots and sera were separated. This took about 30 min. more, so that the time from the addition of calcium to the addition of trichloroacetic acid was about 1 hr. The clots and sera were extracted separately.

It is known that when platelet-rich plasma clots, the platelets become entangled in the clot and sediment with the clot on centrifuging. Therefore the amount of phosphorus left in the platelets after clotting was: phosphorus found in the clot formed in platelet-rich plasma minus phosphorus found in the clot formed in platelet-poor plasma. The difference between this value and phosphorus in the platelets before clotting represented phosphorus lost from platelets during clotting.

Table 8 shows the concentration of phosphorus in the extracts and residues of platelets, clots and sera. The results are expressed as $\mu\text{mg. atoms}$ of phosphorus corresponding either to 1 ml. of dialysed plasma or to the platelets which were present in 1 ml. of dialysed plasma. The gains and losses of phosphorus by platelets and sera are represented by the columns of Fig. 3.

The results were as follows: (1) Almost as much acid-soluble phosphorus was lost by the platelets as was gained by the serum. This was reasonable in view of results already mentioned, which suggested that although some ATP phosphorus was transferred to the residue, if clotting continued for more than 30 min. as it did in this experiment, this phosphorus once more became acid-soluble. (2) Platelets neither gained nor lost extractable lipid phosphorus, whereas the plasma lost a great deal. (3) Platelets lost a little phosphorus associated with the residue, and the residue of the plasma (or serum) gained very much more. The conclusion was that during clotting a considerable amount of lipid phosphorus in the plasma became attached

Table 8. *Concentrations of phosphorus in the sera, clots and platelets of plasma clotted without and with platelets*

Results are expressed as $\mu\text{mg. atoms}$ of phosphorus/ml. of plasma for sera and clots and in $\mu\text{mg. atoms}$ of phosphorus/platelet content of 1 ml. of plasma for platelets. Total P in 1 ml. of platelet-rich plasma was 1680 $\mu\text{mg. atoms}$.

	P soluble in acid and water			Lipid P	Residue P	Total P recovered
	Total P	Inorganic P	P ^B + P ^V of ATP			
Platelets	171	19	52	119	23	1590 (95%)
Clot formed in platelet-poor plasma	13	Nil	Nil	23	23	
Corresponding serum	84	55	Nil	891	245	
Clot formed in platelet-rich plasma	58	16	10	142	23	1620 (96%)
Corresponding serum	219	139	Nil	774	400	

to the residue in the serum in such a way that it could no longer be extracted by chloroform-methanol. Moreover, since the increase in residue phosphorus occurred in the serum and not in the clot, the lipid phosphorus had apparently attached itself to a serum protein other than fibrin or other proteins associated with fibrin in the clot.

Other experiments of this kind confirmed these conclusions. However, in two of them part of the extra phosphorus which became associated with the residue of the serum was derived from lipid

phosphorus in the platelets. The results of one experiment, in which undialysed plasma was allowed to clot for 15 min., are given in Table 9. The recoveries of phosphorus were good, and by comparing the figures for the fractions of platelet-rich plasma with those of platelet-free plasma, the following conclusions could be drawn. During clotting the serum lost 320 μ mg. atoms of lipid phosphorus. The loss from the platelets [given by $229 - (245 - 71)$] was 55, making the total loss of lipid phosphorus 375 μ mg. atoms. The gain of residue phosphorus by the platelets was nil and by the serum 413 μ mg. atoms. The difference, $413 - 375 = 38$ μ mg. atoms of phosphorus, must have come from acid-soluble phosphorus.

DISCUSSION

Some time ago platelets were found to contain remarkably high concentrations of ATP (Born, 1956*a*) and the problem arose whether any particular functions could be assigned to platelet ATP which might be relevant to the physiological functions of platelets.

One line of evidence suggests that ATP may be concerned in the accumulation and retention of 5-hydroxytryptamine and adrenaline by platelets (Born *et al.* 1956; Born & Hornykiewicz, 1957; Born & Gillson, 1957). The observation that the onset of clotting in platelet-rich plasma brings about the rapid disappearance of ATP from the platelets raised the possibility that platelet ATP might also have a function in the process of blood clotting.

The results presented in this paper suggest that, when clotting begins in platelet-rich plasma, each mole of ATP in the platelets loses more than 1 mole but not more than 2 moles of phosphate and that this phosphate becomes temporarily attached to the residue which is left after plasma has been thoroughly extracted with trichloroacetic acid and

Fig. 3. Changes in the concentrations of total phosphorus in the trichloroacetic acid extract, chloroform-methanol extract and residue, of platelets and plasma (serum) before and after clotting. The experiment is described in the text. Filled-in columns represent platelets; open columns represent plasma (serum).

Table 9. *Decrease in lipid phosphorus in plasma and platelets, and increase in residue phosphorus in plasma, during clotting of platelet-rich plasma*

Amounts correspond to 1 ml. of the original plasma.

Conditions	Source	Lipid P (μ mg. atoms)	Residue P (μ mg. atoms)
No clotting	Platelet-rich plasma	2580	852
Clotting in the absence of platelets	Platelets (removed before clotting)	229	24
	Clot	71	17
	Serum	2330	819
		2630	860
Clotting in the presence of platelets	Clot	245	41
	Serum	2010	1232
		2255	1273

with fat solvents. Moreover, shortly after ATP begins to disappear from the platelets some of the phospholipids present in plasma and platelets become less extractable by chloroform-methanol and they too become associated in some way with the residue.

The changes in the concentrations of acid-soluble and lipid phosphorus were small, both absolutely and also in relation to the total amounts of phosphorus present in the extracts. The question arises whether the extraction procedures were adequate for removing acid-soluble and fat-soluble phosphorus in turn from the plasma.

Trichloroacetic acid is in general use for extracting acid-soluble phosphorus from tissues and for precipitating their proteins at the same time. Norberg & Teorell (1933) and Fawaz, Lieb & Zacherl (1937) used trichloroacetic acid for this purpose with blood, before extracting the precipitate with ethanol and ether to obtain the phospholipids. In the experiments here reported two extractions were made with the acid. Then the precipitate was washed twice with water before the phospholipids were extracted. It is unlikely that any acid-soluble phosphorus was left behind.

Similarly, it is likely that two extractions with chloroform-methanol removed all phospholipid except that bound tightly in some way to protein. Thus Table 9 shows that the amount of lipid phosphorus extracted from human plasma was 79.8 $\mu\text{g./ml.}$, which corresponds to nearly 2 mg. of phospholipid/ml. Others have found very similar concentrations of phospholipid in human plasma: Bloor (1921) found 1.92 mg. of phospholipid/ml.; Erickson, Avrin, Teague & Williams (1940) found 1.89 and Taurog, Entenman & Chaikoff (1944) found 2.35 mg./ml.

The residue which is left after extracting plasma with trichloroacetic acid, water and chloroform-methanol is mostly protein. The changes described in this paper suggest therefore that when plasma clots in the presence of platelets, both acid-soluble phosphorus and phospholipid are bound to protein and that a phospholipoprotein is formed. The possible relevance of this to the process of clotting is that the course in time of the formation and breakdown of this phospholipoprotein is very similar to that of plasma 'thromboplastin' (Biggs, Douglas & Macfarlane, 1953). Moreover, when plasmas from different people clot, the amounts of 'thromboplastin' formed vary greatly (Macfarlane, 1956); this may be correlated with the observed variability in the amount of phosphate which is transferred from platelet ATP and from phospholipid to the residue in different plasmas. Little 'thromboplastin' is formed in clotting plasma when platelets are few or absent (Hardisty & Pinniger, 1956), and in the absence of platelets

there was no significant increase in the amount of phosphorus in the residue. It may therefore be suggested that the changes described in this paper could represent the formation and breakdown of 'thromboplastin' in plasma.

SUMMARY

1. Platelets in the blood of man, pig and rabbit contain high concentrations of adenosine triphosphate.

2. When citrated plasma, rich in platelets, is obtained from man or pig and recalcified at 37°, the adenosine triphosphate in the platelets rapidly breaks down. Between 1 and 2 moles of phosphate are transferred from each mole of adenosine triphosphate to the residue left after serum has been extracted successively with aqueous trichloroacetic acid, water and chloroform-methanol.

3. During clotting of platelet-rich plasma the amount of phosphorus extractable from plasma by chloroform-methanol also decreases, and the phosphorus in the residue increases by approximately the same amount. These changes are maximal from 10–30 min. after calcium is added. Later the amount of phosphorus decreases in the residue and increases in the trichloroacetic acid extract.

4. These changes are slight or absent when plasma clots without platelets.

5. The results suggest that when platelet-rich plasma clots, a phospholipoprotein is formed which is later broken down. Various considerations suggest that this material may be plasma 'thromboplastin'.

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The Estimation of Free Sugars in Skeletal Muscle of Codling (*Gadus callarias*) and Herring (*Clupea harengus*)

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Data relating to free sugars in the skeletal muscle of fishes are scarce. Moreover, many of the early reductometric studies (e.g. MacPherson, 1932) lacked specificity and it is now well-established that large excesses of amino nitrogen such as occur in extracts of fish muscle, cause serious inaccuracy with such procedures (Hewitt, 1938; Strange, Dark & Ness, 1955). Tarr (1954) has described the chromatography of concentrated ethanolic extracts, followed by direct photometry of the sprayed papers to evaluate sugars in the flesh of some species of the Canadian Pacific seaboard. In this Laboratory, both interference by 'salt' and fat and low recoveries of ribose accompanied a similar manipulation of extracts of codling and herring muscle. The present paper shows that the loss of ribose results from Maillard (1912) type reactions with amino nitrogen.

In attempting to overcome these difficulties use had been made of a number of techniques employed in current biochemical practice. The removal of salts by electrodialysis (Consden, Gordon & Martin, 1947) and ion-exchange resins is a commonplace in the literature. Partridge (1948) used the latter principle to remove substances interfering with the chromatography of sugars in apple juice but Hulme (1953) demonstrated that strongly basic resin in the OH^- form employed (in conjunction with strongly acid resin in H^+ form) limited the recovery of glucose. Phillips & Pollard (1953) favoured a resin mixture containing strongly basic resin in CO_3^{2-} form, which did not degrade glucose. Strange *et al.* (1955) in a study reported while the

present work was in progress, removed the amino acids of casein hydrolysate by a mixture containing a weakly basic resin in OH^- form before the reductometric determination of glucose in media.

This paper describes the effects of various procedures on the recovery of free sugars from extracts of muscle and reports the danger of serious artifacts which may have relevance to sugar analysis in other biological extracts and fluids. A short preliminary communication of some of the work has appeared (Jones, 1956a).

EXPERIMENTAL

Materials

Codling. These were landed alive from R.V. 'Keelby' and maintained in aerated sea water until killed by a blow on the head. For post-mortem studies the fish were gutted and packed in ice at an ambient temperature of 2.5° . Sterile codling muscle was dissected from the anterior-dorsal part of the fillet, immediately after death, under aseptic conditions: for studies on autolysis, samples of muscle (5 cm. $\times$ 2 cm. $\times$ 1 cm.) were stored at 0° in sealed Petri dishes.

Herring. These fish, 14–20 hr. dead and stored at about 0° , were made available through the kindness of the Marine Laboratory, Scottish Home Department, Aberdeen.

Resins. Amberlite resins IR-120, IR-4B (Rohm and Haas Co., Philadelphia, Pa., U.S.A.); Dowex 50; Dowex 2 (Dow Chemical Co.); sulphonated polystyrene, nominal divinyl content 4.5% (PSX), De-Acidite FF (Permutit Co. Ltd.). The resins were used in the form and mesh-size reported in the text.

Nucleotides, ribosides and sugar phosphates. Adenosine mono-, di- and tri-phosphates were supplied by the Sigma