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IDENTIFICATION OF ALGOGENIC SUBSTANCES IN HUMAN ERYTHROCYTES

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SUMMARY

1. Lysates of human erythrocytes produce pain when applied to a human blister base. The algogenic material is not potassium, acetylcholine, bradykinin, 5-hydroxytryptamine, histamine or a prostaglandin, and is dialysable.

2. Fractionation of dialysates of freshly lysed erythrocytes by Sephadex gel filtration coupled with assays on the human blister base preparation showed that the algogenic material was a mixture of the adenyl compounds adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP).

3. On the human blister base preparation ATP, ADP and AMP had comparable activity and produced threshold pain in a concentration of 2 μM.

4. The rabbit isolated jejunum preparation was found useful in these studies since ATP, ADP and AMP produced a relaxation which was proportional to their concentration in test samples obtained from dialysates. Of more limited usefulness was the rat isolated stomach strip preparation on which ATP and ADP produced contractions which also were proportional to their concentrations in text samples.

5. The possible role of adenyl compounds in the production of pain *in vivo* is discussed.

INTRODUCTION

In 1964 Keele & Armstrong reported that lysates of human erythrocytes produced pain when applied to a human blister base. The haemolysates studied also contained potassium (K^+) in algogenic concentrations (Keele & Armstrong, 1964), generally of the order of 16 mM, but the pain produced by the haemolysates was appreciably greater than that caused by K^+ alone. Furthermore, lysates obtained from cat erythrocytes which had a much lower (non-algogenic) concentration of K^+ also produced pain.

From these findings Keele & Armstrong concluded that human erythrocytes in general contain algogenic material other than K^+.

In subsequent studies (unpublished in detail but referred to by Keele & Armstrong, 1968) the following observations were made. The algogenic material was soluble in water, methanol and to some extent ethanol but insoluble in other organic solvents, it behaved as an anion on ion-exhange chromatography and it was unstable when boiled at low pH for 1–2 hr. It was of small molecular size since it was dialysable through membranes with a pore diameter of 4·8 nm and dialysates containing the algogenic material relaxed the rabbit isolated jejunum preparation and contracted the rat isolated stomach strip preparation. These actions were not affected by concentrations of atropine or antihistamine drugs which markedly reduced responses to acetylcholine and histamine. Treatment of dialysates with chymotrypsin also had no effect on the responses of the two preparations to them.

From these findings and the observation that the algogenic material was insoluble in ether at pH 3, and assuming that the same substance(s) was (were) responsible for the production of pain and for the effects on the two isolated organ preparations, it was concluded that the algogenic material was not acetylcholine (ACh), histamine, a prostaglandin or bradykinin. Studies with 5-hydroxytryptamine (5-HT) antagonists showed that 5-HT, another known algogen (Keele & Armstrong, 1968; Lim, 1968), also could be ruled out, provided that lysates were made from freshly collected blood. 5-HT, however, was present in algogenic concentrations when haemolysates were prepared from outdated blood samples.

This paper describes the results of further studies which led to the identification of adenine nucleotides as the algogenic material in human erythrocytes.

METHODS

Blood was taken from an antecubital vein of healthy human subjects of either sex and collected in bottles containing ACD anticoagulant solution (U.S.P. formula A – citric acid, 0·04 M; sodium citrate, 0·1 M; dextrose, 0·12 M), using 75 ml. of the solution for 500 ml. blood. The blood was centrifuged in polyethylene containers for 30 min at 4° C and 165 *g*, and the plasma and white cell layer were withdrawn by suction. The remaining erythrocytes were washed twice with an equal volume of ice-cold 0·15 M-NaCl solution. After the second washing the top quarter volume of erythrocytes was also siphoned off and discarded. Since the algogenic material was dialysable, the studies on its identity and actions were based on dialysates of the lysed erythrocytes.

Preparation of dialysates. To achieve haemolysis an equal volume of ice-cold distilled water was added to the washed erythrocytes, and the samples were then subjected to rapid freezing at − 32° C, followed by thawing. The haemolysate was transferred to washed Visking dialysis tubing (inflated diameter 1·9 cm; average pore diameter 4·8 nm) which was submersed in an equal volume of distilled water and

shaken for 24 hr at 4° C. The dialysis was repeated for a second 24 hr period and the two dialysates were combined. The weight of solid material in the (combined) dialysate ranged from 6 to 8 mg/ml. erythrocytes ($n = 6$). The algogenic material was chloroform-insoluble and dialysates were partly purified by shaking them in a dried state in redistilled chloroform (1 ml. chloroform/6–8 mg dialysate) at room temperature for 1 hr. The chloroform was filtered off and discarded and the process repeated twice on the residue.

Molecular sieve chromatography. To separate dialysates into fractions they were subjected to column chromatography. Glass columns (130 cm long, 1 cm in diameter) were packed with the cross-linked dextran gel Sephadex G-15 or G-10 (Pharmacia, Sweden). Distilled water was passed through the column until the eluate was free of U.V. absorbing substances. Ten mg of blue dextran (Dextran 2000, Pharmacia) dissolved in 0·1 ml. distilled water were loaded onto the column and eluted with 0·1 M-NaCl to check the uniformity of packing and to determine the void volume. Dialysates and other material were eluted from the column with distilled water at a flow rate of 15–20 ml./hr. The eluates were collected in consecutive 1 ml. fractions using an automatic fraction collector.

Charcoal extraction. Purine and pyrimidine compounds are selectively adsorbed from salt-containing solutions by activated charcoal. Following adsorption they can be eluted with ethanolic ammonia which can be removed by evaporation. This was made use of in the following way. Norit A charcoal was made phosphate free as described by Crane (1958), and then washed with ethanolic ammonia (ethanol, 8·6 M; NH_4OH, 2·7 M) and dried at 110° C. The charcoal was added to redissolved chloroform purified dialysate or solutions of adenyl compounds. One to 2 mg charcoal were required for complete adsorption (on to the charcoal) of the algogenic material in 1–2 mg of dried chloroform purified dialysate or of 0·14 μmole of adenyl compounds, as calculated from measurements of U.V. absorption. In practice, the charcoal-dialysate or charcoal-adenyl compound samples were acidified with HCl (final concentration 0·05 M), and after shaking for 30 min at room temperature centrifuged. The charcoal then was washed twice with 0·05 M-HCl, using one tenth of the volume of the initial sample. The adsorbed material was eluted by suspension of the charcoal in ethanolic ammonia (volume equal to initial sample volume), shaking for 30 min in a stoppered container and centrifuging. The supernatant and those of two subsequent washings (with distilled water in one tenth of the volume of the initial sample) were combined and evaporated to dryness.

Perchloric acid (PCA) extraction. To measure the concentrations of purine nucleotides in protein containing solutions, the proteins must be removed and this was done by PCA extraction. Erythrocytes or haemolysates were added to an equal volume of ice-cold 0·6 M-PCA, mixed well and centrifuged at 5° C and 2000 *g* for 10 min. The supernatant, which contained the nucleotides, was neutralized immediately with 2·5 M-KOH and placed in an ice bath for 10 min. This precipitated the excess of PCA as its K salt, which was then removed by filtration. Filtrates were frozen at $-32°$ C until assayed.

Paper chromatography. Whatman no. 1 paper was washed by descending chromatography with a solution consisting of 9 parts redistilled methanol and 1 part 11·6 M-HCl, using 25 ml. for each paper. The washed paper was dried and then washed with distilled water till acid-free. Test samples, dissolved in distilled water, were applied to the paper either in spots or bands and run by ascending flow in various solvent systems, details of which are given under Results.

The dried chromatograms were scanned for areas of U.V. absorption by viewing under a U.V. lamp with high emission in the range of 250–280 nm (Hanovia 'chromatolite'). For visualizing individual components chromatograms were sprayed

with ninhydrin, phosphate, silver nitrate (for Na^+ and K^+) and silver nitrate-bromophenol blue reagents (for purine and pyrimidine bases) (Smith, 1960).

Analytical methods

Na and K concentrations were determined with an EEL flame photometer.

For *U.V. absorption and colorimetric measurements* an automatic scanning spectrophotometer (Unicam-Model SP 800) was used. Spectral measurements were made in a 1 cm light-path.

Measurement of phosphates. Free (inorganic), acid-labile and stable organic phosphates were determined by the method of Fiske & Subbarow (1925). This method depends on the finding that free phosphate in acidic solution reacts with molybdate to give phosphomolybdic acid, which, on reduction by 1-amino-2-naphthol-4-sulphonic acid in the presence of sulphite yields a blue dye. Measuring at 660 nm the colour intensity of samples treated in this way gives the content of free inorganic phosphate. When samples are heated gently at 100° C for 7–15 min in acidic solution, the so-called acid labile phosphate is released from compounds which have a labile phosphate bond (e.g. ATP and ADP), while heating at 150–160° C for 3 hr also hydrolyses the more stable organic phosphate bonds, e.g. of AMP. From the colour reaction in the heated samples values for acid-labile and stable organic phosphates are obtained.

Pentose was measured by the method described by Brown (1946). The principle of the method is that pentose reacts with orcinol (3,5-dihydroxy-toluene) in acidic solution containing ferric ammonium sulphate, to produce a blue coloured compound which can be measured at 660 nm.

Enzymic estimations of nucleotides

AMP and ADP were determined by the method described by Adam (1965). This method is based on a series of enzyme catalysed reactions which for the last step require nicotinamide-adenine dinucleotide (NADH), the concentration of which is determined by measuring absorption at 360 nm. To a sample containing ADP and/or AMP are first added phosphoenol pyruvate, NADH and ATP. On subsequent addition of pyruvate kinase, ADP is phosphorylated to ATP while phosphoenol pyruvate is converted to pyruvate. Finally, on addition of lactic dehydrogenase pyruvate is reduced with the oxidation of NADH to NAD^+. From the absorption change at 360 nm the concentration of ADP can then be calculated. On completion of the reaction, addition of myokinase converts AMP to ADP and this starts again the sequence of reactions described. From the absorption change associated with this the concentration of AMP present in the sample is then calculated. A Boehringer kit (catalogue no. 15980 TAAB) containing the individual enzymes and co-factors was used.

ATP was measured by a modification of the method described by Strehler & McElroy (1957). The principle of the method is that when ATP is mixed in the presence of air (O_2) with firefly extract, the relevant constituents of which are luciferin and luciferase, light photons are emitted. The amount of light emitted is proportional to the amount of ATP present and was measured with a spectrophotofluorometer (Aminco Bowman) modified so that the photomultiplier cell was attached directly to the cuvette chamber. The output of the spectrophotofluorometer was recorded with a pen recorder (Devices). For adding the firefly extract to the test sample, a fine stainless steel tube was passed through the light-tight lid of the cuvette chamber. To this was attached a polyethylene tube leading to the bottom of the cuvette. The volume of the test sample in the cuvette was 0·1 ml. After recording the light emission of the test sample, 0·4 ml. ice-cold firefly extract, prepared by

reconstituting the dried extract of 50 mg firefly tails (FLE-50, Sigma Chemical Co.) with 5 ml. distilled water, were injected rapidly into the cuvette through the polyethylene tube. Light emission, which was maximal 10 sec after addition of the extract, was used for calculation of the ATP concentration.

Biological assay preparations

Human blister base preparation. The method was essentially that described by Armstrong, Dry, Keele & Markham (1953) and involves raising a cantharidin blister on the ventral surface of the forearm. After removal of the epidermis, the blister base is continuously bathed in blister Ringer solution with the composition (mM): NaCl, 157; KCl, 5·4; $CaCl_2$, 2·2, $NaHCO_3$, 1·8; the solution was gassed with 95% O_2:5% CO_2 and had a pH of 7·4. Test solutions were applied in a volume of 0·2 ml. for 2 min and a rest period of 8 min was allowed between tests. All tests were blind as far as the subject was concerned. The pain produced by test solutions was recorded by moving a pointer along a scale marked in units. The pointer was connected via pulleys to a lever, the excursions of which were recorded on a slow moving smoked drum. The pain scale used represented no pain (0 units), slight pain (1 unit), moderate pain (2 units), severe pain (3 units) and very severe pain (4 units). Sensations registered as 0·5–1 unit were taken as threshold pain.

All test samples were adjusted with a modified blister Ringer so that the concentration of K^+ and the pH were the same as that in blister Ringer.

Rabbit isolated jejunum preparation. Segments of jejunum were removed from rabbits anaesthetized with Na pentobarbitone, 0·24 m-mole/kg given I.V. They were suspended at 37° C in Krebs solution with the composition (mM) NaCl, 137; KCl, 4·7; $CaCl_2$, 2·5; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $NaHCO_3$, 2·5; glucose, 5·5. The solution was gassed with 95% O_2:5% CO_2. Muscle tone was recorded either on a smoked drum with an isotonic frontal writing lever which was loaded with 1–2 g and gave a fifteenfold magnification, or with an isometric tension transducer (Statham GI 32350) coupled to a pen recorder (Devices). Test samples were added to the organ bath for 0·5–1 min and a rest period of 2–2·5 min was allowed between tests.

Rat isolated stomach strip preparation. A longitudinal strip was prepared from the fundus of the stomach of adult Wistar rats of either sex as described by Vane (1957). It was suspended in Krebs solution with the composition given above. Muscle tone was recorded as described for the rabbit preparation, except that the lever was loaded with 1–1·5 g and test solutions were added for 1·5 min with an interval of 2·5 min between tests.

Control experiments showed that K^+ and Na^+ in the concentrations in which they were present in test samples did not affect the responses of the two isolated organ preparations to the active component(s) of the test samples.

RESULTS

Algogenic action of dialysates of lysed human erythrocytes

Dialysates (chloroform purified), diluted in modified blister Ringer so as to contain K^+ in a concentration equal to that of blister Ringer, regularly produced pain on the human blister base preparation. Threshold pain (0·5–1 unit) was registered with dilutions containing about 0·12–0·25 mg of dried dialysate/ml. More concentrated solutions produced greater pain, but the slope of log concentration of dialysate: pain intensity plots was not very steep, and even the highest concentrations of dialysate which could

be tested, i.e. approx. 1 mg dried dialysate/ml. did not produce severe pain. Fig. 1 shows typical effects obtained with dialysate samples as compared with effects of 5-hydroxytryptamine (5-HT) and acetylcholine (ACh). As can be seen, the algogenic response produced by dialysate samples occurred after a latent period of 15–20 sec and like the responses to 5-HT and ACh was only short lasting.

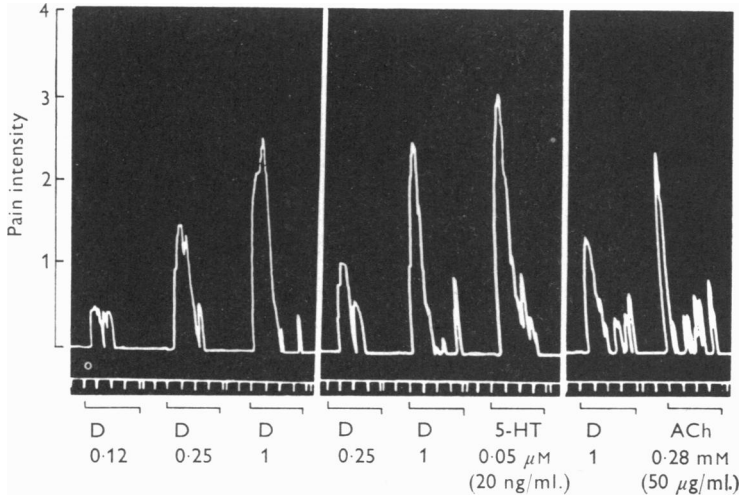

Fig. 1. Algogenic action on the human blister base preparation of dialysate (D), 5-HT and ACh. The figures below D give the amount of chloroform purified dried dialysate in mg/ml. blister Ringer. Application and removal of test samples are represented by └─┐. The time base gives 30 sec intervals; double markings represent periods between tests during which the kymograph was stopped. The three parts of the Figure are parts of three separate experiments in each of which samples were tested at 10 min intervals.

Action of dialysates on isolated smooth muscle preparations

On the rabbit isolated jejunum preparation dialysate produced a transient reduction in the height of spontaneous contractions. The threshold concentration for this effect was 10 μg dried dialysate/ml. bath fluid and the log concentration of dialysate:response plot was steeper than that obtained on the human blister base preparation.

The rat isolated stomach strip, on the other hand, contracted in response to dialysate. The contraction was sometimes preceded by relaxation, and a concentration corresponding to 5 μg dried dialysate/ml. bath fluid regularly produced a recordable contraction. The log concentration of dialysate:response plot also was steeper than that obtained on the human blister base preparation.

Separation of dialysate into biologically active fractions by Sephadex chromatography

A Sephadex G-15 chromatography column was loaded with 100 mg of chloroform purified dried dialysate, dissolved in 1 ml. distilled water, and eluted with distilled water at a flow rate of 20 ml./hr. The eluate was collected in 1 ml. fractions. Each 1 ml. eluate was tested for Na^+ and K^+ content and 0·01 ml. samples of them were subjected to ninhydrin and phosphate spot tests on filter paper. As Fig. 2*A* shows there were two distinct peaks for Na^+, an initial small peak which was followed by a much larger one. K^+ was eluted in a single peak which was slightly delayed relative to the larger Na^+ peak. Ninhydrin reacting compounds gave three peaks, one of which was associated with the small Na^+ peak. The latter also gave a positive test for phosphate.

The large number of eluate samples made analysis of the biological activity of each sample on the human blister base preparation impossible. Therefore tube contents were pooled into six fractions. The first fraction comprised eluates 1–57 which included the void volume, 49 ml.; the other five fractions represented either separate ninhydrin peaks or the salt peak as shown in Fig. 2. Assay of the six fractions on the human blister base preparation showed that the algogenic activity of the dialysate was mainly confined to those fractions which included the small Na^+ and the large Na^+ and K^+ peaks (Fig. 2*B*). According to assays on the rat isolated stomach strip preparation, however, most of the activity was associated with the small Na^+ peak. The total activity recovered on each of the two assay preparations was approximately 50 % of the activity of the dialysate applied to the column. Recoveries of Na^+ and K^+ in the eluates were similarly incomplete and amounted to approximately 60 %.

To prepare fractions large enough for a more detailed analysis 1·5 g chloroform purified dried dialysate were subjected to gel filtration in three separate runs, applying 500 mg dialysate, dissolved in 1 ml. distilled water, to the column for each run. The eluates were pooled into three fractions. The first (fraction A) included the eluates preceding the tube in which the concentration of Na^+ began to rise; the second (fraction B) consisted of the eluates from the end of fraction *A* to the beginning of the large Na^+ peak; and the third (fraction *C*) was composed of eluates containing the major Na^+ and the K^+ peaks.

Analyses of the biological activities of the three fractions showed that fractions B and C, but not fraction A had algogenic activity and that, on a weight basis, the activity of fraction C was slightly greater than that of fraction B. On the rabbit isolated jejunum preparation the results paralleled those on the human blister base except that the relative potencies of

fractions B and C were reversed. On the rat isolated stomach strip preparation fraction B was by far the most active fraction and only traces of activity were present in fraction C; traces of activity were also present in fraction A.

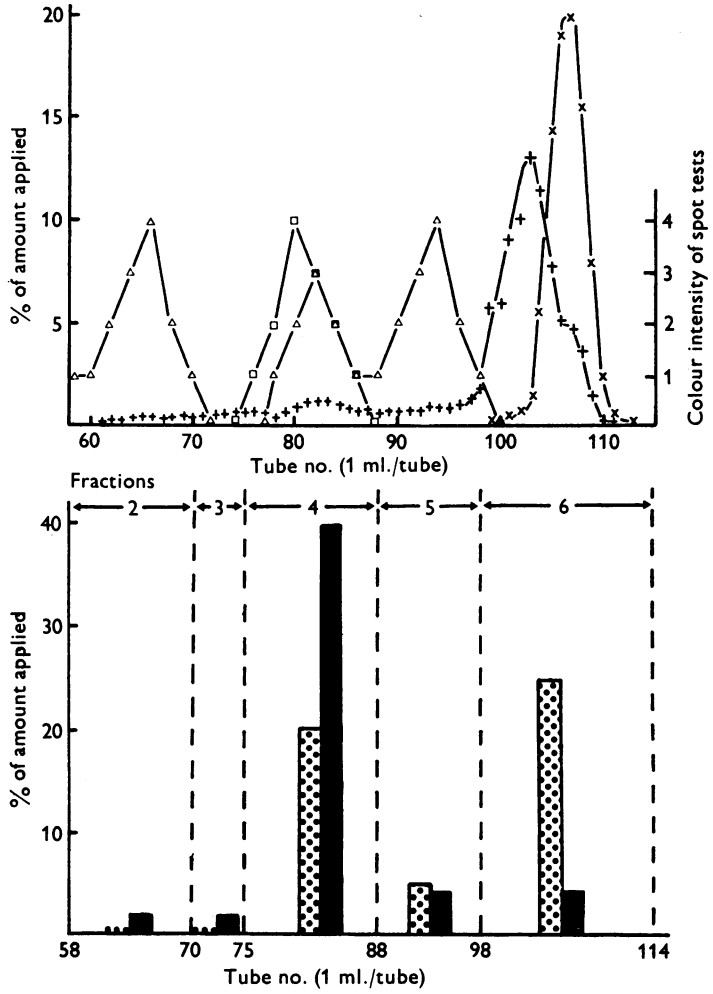

Fig. 2. Analysis of eluates obtained by separation of chloroform purified dialysate (100 mg dissolved in 1 ml. distilled water) on a Sephadex G-15 column. *A*, the concentration of Na^+ (+) and K^+ (×) as % recovered (left ordinate) and the content of ninhydrin reacting (△) and phosphate containing (□) compounds expressed in arbitrary units based on the intensity of spot test colour (right ordinate). *B*, the biological activity of combined eluates (fractions) on the human blister base (dotted columns) and rat isolated stomach strip (solid columns) preparations, expressed as % recovered.

Analysis of fraction C. A sample of fraction C was subjected to further gel filtration on a Sephadex G-15 column and the eluates (consecutive 1 ml. samples) were analysed for U.V. absorption in the range of 200–450 nm, Na^+ and K^+ concentrations and ninhydrin and phosphate reacting compounds. Biological activity, as determined on the rabbit isolated jejunum preparation, was paralleled by U.V. absorption which was maximal at 260 nm, and by a phosphate reaction. The ninhydrin reaction did not parallel the biological activity.

The peaks for Na^+ and K^+ were in the eluates with the highest biological activity. To determine the relationship between Na^+ and K^+ and biological activity fraction C (80 mg applied as bands to three papers) was subjected to paper chromatography in a solvent composed of methanol, 98 ml.; acetic acid, 2 ml. The R_F values, determined by spraying with silver nitrate were 0·45 for Na^+ and 0·25 for K^+. These values are comparable to those published by Smith (1960). The U.V. absorbing component remained near the origin and had an R_F value of 0·05. Assays of eluates of serial strips (of duplicate papers) on the human blister base and rabbit isolated jejunum preparations showed that the total activity of fraction C was confined to the U.V. absorbing area. On a weight basis the amount of material which accounted for the biological activity was 2–3 % of the amount chromatographed.

To establish the relationship between phosphate containing compounds, U.V. absorption and biological activity, a sample of fraction C was extracted in the methanol; acetic acid solvent (as above). The extract was dried and then dissolved in distilled water and subjected to chromatography on a Sephadex G-10 column. The material responsible for biological activity absorbed U.V. light with a maximum at 260 nm and contained pentose and organic phosphate. The molar ratio of pentose to organic phosphate was approximately 1.

These findings suggested that the biological activity of fraction C was attributable to AMP. Therefore, enzymic estimations of the AMP content of fraction C ($n = 3$) were performed. They gave a value of $0{\cdot}023 \pm 0{\cdot}003$ μmole AMP per mg fraction C. Thus, on a weight basis AMP amounted to about 1 % of fraction C.

On the human blister base and rabbit isolated jejunum preparations AMP had qualitatively the same action as fraction C, and slopes of log concentration: response plots for them were parallel. Based on assays on the rabbit isolated jejunum preparation ($n = 4$; Fig. 3) the AMP content of fraction C was of the order of 1 %. This agrees with the enzymic assay of AMP. AMP had no action on the rat isolated stomach strip preparation in concentrations which were 10 times greater than those which produced a threshold effect on the human blister base and rabbit isolated

jejunum preparations. This explains why fraction C only produced marginal effects on the former.

Analysis of fraction B. A sample of fraction B was subjected to further gel filtration on Sephadex G-15 and tests on the eluates gave the following results. Biological activity, determined on the rabbit isolated jejunum preparation, was associated with a U.V. absorption which was maximal

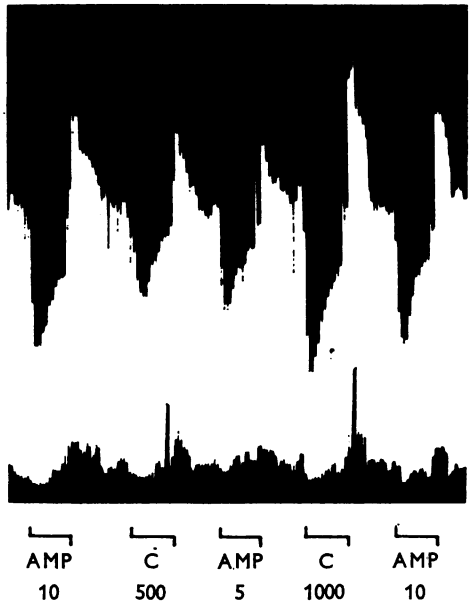

Fig. 3. Assay of AMP and fraction C (C) on the rabbit isolated jejunum preparation. The figures are μg added to the organ-bath (10 ml.); 10 μg AMP = 23 n-mole. Test solutions were added for 1 min ⌐¬ at 3 min intervals. The kymograph was switched off at the end of each test period and restarted 30 sec before a test.

at 248–250 nm. Spot testing of aliquots of the biologically active eluates with a range of reagents for a variety of chemically reactive groups showed that only the test with silver nitrate-bromophenol blue reagent, which gives a blue colour with purine or pyrimidine bases, paralleled the distribution of both biological activity and U.V. absorption.

Substances which are erythrocyte constituents and have a U.V. absorption maximum at the appropriate wave-length are guanosine (252 nm) and inosine (248–249 nm) derivatives (Volkin & Cohn, 1954). Biological assay of these substances on the rabbit isolated jejunum preparation showed that on a weight basis their activity was less than that of fraction B. Taking also into consideration their concentration in erythrocytes

(Bishop, Rankine & Talbott, 1959; Mandel, Chambon, Karon, Kulic & Serter, 1961–62), guanosine and inosine derivatives, therefore, could be ruled out as substances accounting for the biological activity of fraction B.

Aliquots of fraction B then were subjected to paper chromatography in a solvent composed of *n*-butanol 120 ml., glacial acetic acid 30 ml., distilled water 50 ml., using various adenosine, guanosine and inosine derivatives as standards. After drying, the papers were scanned for U.V. absorbing areas. There were several such areas. They were eluted and assayed on the rabbit isolated jejunum preparation. One area with an R_F of 0·12 had the highest absorption intensity which was maximal at 250 nm. However, it had no biological activity. Most of the biological activity recovered (75%) was associated with an area which had an absorption maximum at 260 nm and an R_F value of 0·02–0·05. Standards with comparable R_F values were ATP and ADP.

TABLE 1. Charcoal extraction of chloroform purified dried dialysate

	Composition of dialysate subjected to charcoal extraction	Distribution of substances between	
		Supernatant	Eluate
Dry weight (mg)	1000	914 (91)	117 (12)
Na^+ (μmole)	6400	4860 (76)	70 (1)
K^+ (μmole)	5600	4320 (77)	20 (<0·5)
U.V. absorption at 260 nm	1157	21 (2)	773 (67)
Pentose (μmole)	104	26 (25)	68 (66)
Phosphate (μmole):			
Inorganic phosphate	194	180 (93)	2 (1)
Acid-labile phosphate	62	23 (37)	8 (13)
Stable organic phosphate	125	34 (27)	55 (44)
Total phosphate	381	237 (62)	65 (17)
Biological activity on rabbit isolated jejunum	Taken as 100%	Contractile response	(75)*

Dialysate was treated with charcoal as described under Methods. Material not adsorbed on to the charcoal is given in the column headed supernatant. Material which was adsorbed and then eluted with ethanolic ammonia is given in the column headed eluate. The figures in parentheses are percentages of the values for the dialysate. Relaxation (*) but not contractile responses show parallelism with pain producing activity.

Quantitative analysis of adenyl compounds in dialysates

The results described so far indicate that adenine nucleotides account for the algogenic activity of dialysates. These compounds can be adsorbed on to activated charcoal and, therefore, the following experiments were carried out to obtain further information. One gram of chloroform

purified dried dialysate was subjected to charcoal adsorption and the charcoal was subsequently extracted with ethanolic ammonia as described under Methods. Table 1, which summarizes the results obtained, shows that all the biological activity of dialysates on the rabbit isolated jejunum preparation which showed a parallelism to the algogenic activity on the human blister base preparation, i.e. the material which caused relaxation, was adsorbed on to charcoal and 75% of it was eluted from the charcoal. In control experiments with adenosine, AMP, ADP and ATP the recovery yields were 86, 67, 56 and 49%, respectively. The charcoal extract had 12% of the dry weight of the dialysate and only small amounts of Na^+ and K^+ were adsorbed into charcoal. The amount of adenine nucleotides in the charcoal extract, calculated from the U.V. absorption at 260 nm and the molar extinction coefficient, was 50 μmole. A comparable result was obtained in analyses of the stable organic and acid-labile phosphate content of the charcoal extract. They gave values of 55 and 8 μmole, respectively, on the assumption that the source of the former was AMP and that of the latter ADP or ATP or a mixture of both. Justifying this assumption is the finding that the pentose content of the charcoal extract was 68 μmole.

Gel filtration on a Sephadex G-15 column of 100 mg of the dried charcoal extract, redissolved in 1 ml. distilled water, gave eluates which had six peaks of U.V. absorption. These peaks were numbered in the order in which they were eluted from the column. Peaks 1, 2, 4 and 6 had absorption maxima at 260 nm, while the absorption maxima of peaks 3 and 5 were at 250 nm. Paper chromatographic analysis of the six peaks in a solvent system described by Krebs & Hems (1953), which separates nucleosides and their phosphorylated derivatives (namely iso-butyric acid, 100 ml.; ammonia (sp. gr. 0·88) 18 M, 4·2 ml.; Versene, 0·1 M, 1·6 ml.; distilled water, 55·8 ml.; final pH 4·6), gave the following result. Peaks 1, 2, 4 and 6 had R_F values which corresponded to those of ATP, ADP, AMP and adenosine, respectively. Peak 3 mimicked a mixture of inosine monophosphate and AMP and peak 5 had the same R_F value as inosine. Assay of the eluates by specific enzymic methods for adenyl derivatives and biological assay on the rabbit isolated jejunum preparation gave the following results. The total amounts of ATP, ADP and AMP determined enzymically were 0·9, 3·2 and 23·7 μmole, respectively, and those determined by bio-assay were 1·7, 3·3 and 23·8 μmole, respectively. With respect to ADP and AMP the agreement is good. The discrepancy between the ATP results could be due to an interference by anions with the enzymic assay, since it was found that chloride ions reduce values of standards by 30% and 90% in 0·15 and 1·5 M chloride concentration, respectively. Studies of the distribution of ATP, ADP and AMP between eluates (see Fig. 4) showed that their peaks corresponded to peaks 1, 2 and 4,

respectively, as located by U.V. absorption. Peaks 1 and 2 are in eluates which are components of fraction B and peak 4 is in eluates which make up fraction C. It can be concluded, therefore, that ATP and ADP are the active components of fraction B and AMP is the active component of fraction C. Adenosine which was responsible for peak 6 of the U.V. absorption was present only in amounts which were less than those required to produce a biological effect.

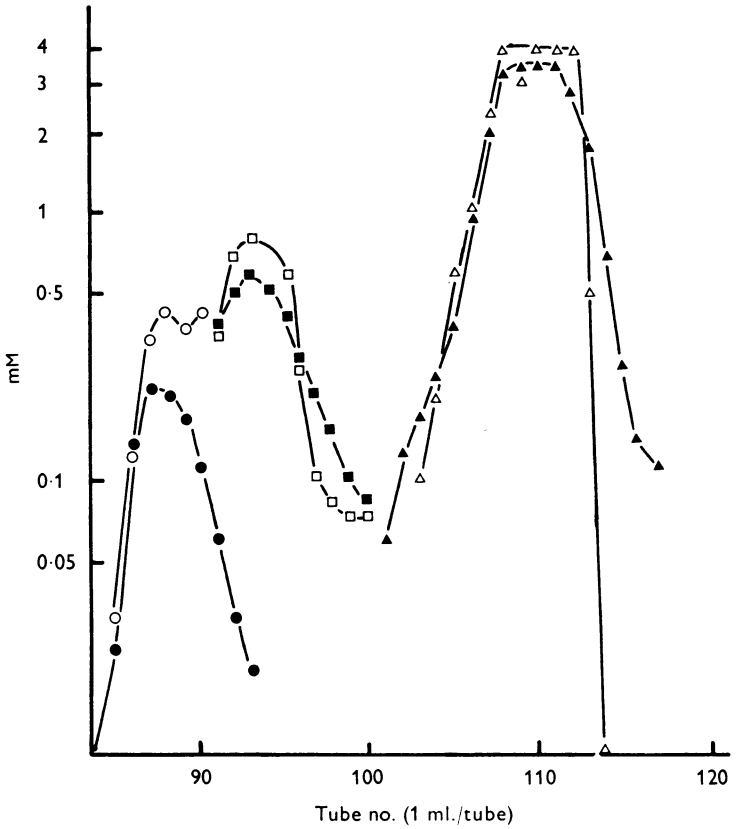

Fig. 4. Separation on a Sephadex G-15 column of 100 mg of charcoal extract obtained from chloroform purified dialysate. The concentration (mM) of ATP (●, ○), ADP (■, □) and AMP (▲, △) were determined by enzymic methods (filled symbols) and by bio-assay on the rabbit isolated jejunum preparation (open symbols).

Consistent with this conclusion are results obtained when 1 ml. of a model solution containing 2 mg of each of the adenyl compounds (i.e. 7·5, 4·7, 3·7 and 3·2 μmole adenosine, AMP, ADP and ATP, respectively) and 100 mg of both NaCl (1·7 m-mole) and KCl (1·3 m-mole) was subjected

to gel filtration on a Sephadex G-15 column. The eluates had four peaks of U.V. absorption. Aliquots from the tubes with the maximum U.V. absorption in each peak, together with the appropriate standards then were subjected to paper chromatography in the solvent system of Krebs & Hems (1953; see above). Using U.V. absorption and phosphate tests for identification it was found that peak 1 was a mixture of ATP and ADP, peak 2 was ADP, peak 3 was AMP and peak 4 was adenosine. Na^+ and K^+

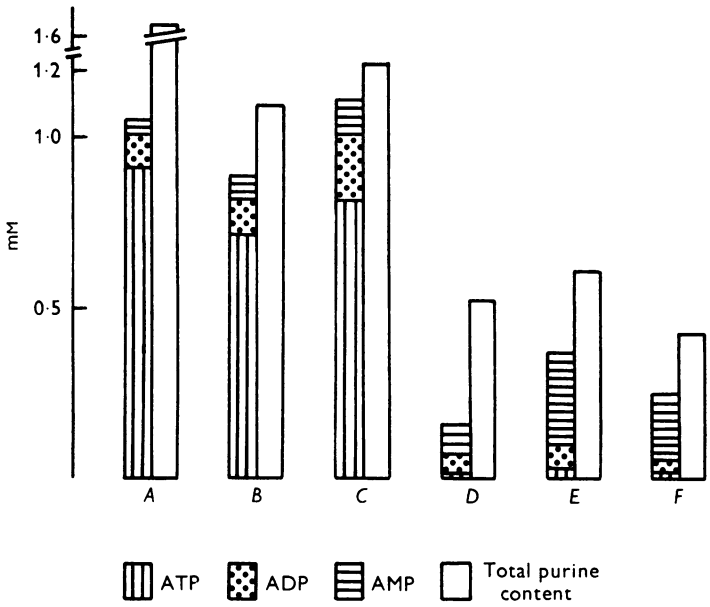

Fig. 5. Concentrations of ATP, ADP, AMP and total purines in erythrocytes, calculated from analyses of whole blood (*A*), washed erythrocytes (*B*), haemolysate (*C*), haemolysate after dialysis (*D*), dialysate (*E*) and charcoal extracted dialysate (*F*). For calculating the values for *D*, *E* and *F* the assumption was made that during dialysis the purines equilibrate across the dialysis membrane. The nucleotides were estimated enzymically and the value for total purine content was obtained from U.V. absorption measurements.

were eluted in close proximity to peak 3. Recoveries, calculated from the U.V. absorption of the eluates, amounted to 98 % for ATP and ADP when determined jointly, 104 % for AMP, 103 % for adenosine and 100 % each for Na^+ and K^+.

Concentrations of adenine nucleotides in erythrocytes, haemolysates and dialysates

Freshly collected erythrocytes contain ATP in a concentration of 0·8–1 mM, while ADP and AMP are present in approximately 0·1 and 0·01 mM

concentration, respectively (Bishop *et al.* 1959; Mandel *et al.* 1961–2). In the dialysate and the various preparations obtained from it, the relative concentrations of ATP, ADP and AMP are reversed. Calculating concentrations in erythrocytes from the results obtained with charcoal extracted dialysate gives values of 0·02, 0·03 and 0·24 mM for ATP, ADP and AMP, respectively. Correcting these figures for losses incurred subsequent to the preparation of the dialysate would raise them by less than

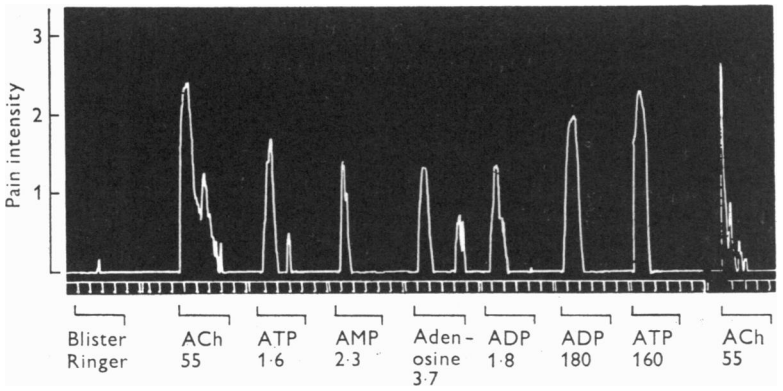

Fig. 6. Algogenic action on the human blister base preparation of ATP, ADP, AMP, adenosine and ACh. The figures are concentrations (μM) in blister Ringer. Applications and removal of test samples are represented by ⌊⌋. The time base gives 30 sec intervals; double markings represent periods between tests during which the kymograph was stopped. Testing was at 10 min intervals.

50% and would not change the ratios between the nucleotides. To determine at what stage the marked loss of the higher phosphorylated compounds occurred, the following study was undertaken. Erythrocytes, prepared from 500 ml. freshly collected blood, were lysed and the haemolysate was dialysed. The dialysate was then extracted with charcoal and the charcoal eluted. At the end of each preparative stage a 5 ml. aliquot of material was taken and immediately deproteinated by PCA extraction. The concentrations of ATP, ADP and AMP were then determined by the enzymic methods. In addition, U.V. absorption at 260 nm was measured and used as a measure of purine content. The samples analysed were whole blood, washed erythrocytes, haemolysate, dialysate, haemolysate remaining in the dialysate bag at the end of the dialysis period, and the charcoal extract of the dialysate. The results, summarized in Fig. 5, show that the values for individual adenine nucleotides in fresh blood and washed erythrocytes were similar to published values; it was during dialysis when the values for ATP decreased markedly while those for AMP showed the reverse.

Algogenic action of adenyl compounds on the human blister base preparation

The application of solutions of ATP, ADP, AMP or adenosine to the blister base elicited after a latent period of 10–20 sec pain which lasted for up to 100 sec, with the highest concentrations producing the longest lasting effects. Threshold concentrations were of the order of 2 μM (i.e. about 1 μg/ml.) for all four compounds and the degree of pain which was produced with the highest concentrations used was between moderate and severe, i.e. 2·5 units of pain were recorded with concentrations of the order of 200 μM. Typical responses are shown in Fig. 6.

DISCUSSION

The results reported in this paper show that the adenyl compounds ATP, ADP and AMP are the material in erythrocytes which produces on the human blister base preparation pain that cannot be accounted for by potassium according to observations by Keele & Armstrong (1964). In erythrocytes the concentration of ATP is considerably higher than those of ADP and AMP. On the human blister base preparation the three adenyl compounds have comparable algogenic activity and, therefore, the pain produced by freshly prepared haemolysates would be mainly caused by ATP. Storage of erythrocytes for 24–48 hr has only a small effect on the relative concentrations of individual adenine nucleotides (Dern, Brewer & Wiorkowski, 1967). The experiments reported in this paper show that subjecting haemolysates to dialysis leads to a considerable loss of ATP, a marked increase in the concentration of AMP and an over-all loss of phosphorylated derivatives of adenosine, whereas the total purine content after dialysis was comparable to that of the haemolysates before dialysis. The reason for this must be that haemolysis causes a breakdown of the compartmentalization between adenine nucleotides and phosphatases (Scharff & Vestergaard-Bogind, 1966). The algogenic action of dialysates, therefore, is mainly accounted for by AMP.

The adenyl compounds produce pain in concentrations as low as 2 μM but the slope of log concentration:pain intensity plots is not very steep. If the same applies at other sites it is unlikely that the adenyl compounds alone can produce severe pain *in vivo*. Release of adenyl compounds has been shown to occur on stimulation of isolated muscle and nerve tissues (Abood, Koketsu & Miyamoto, 1962; Kuperman, Volpert & Okamoto, 1964; Boyd & Forrester, 1968; Burnstock, 1972; McIlwain, 1972; Silinsky, 1975). Studies in man show that muscular exercise increases the venous plasma concentration of ATP (Forrester & Lind, 1969) and of ADP and AMP (Parkinson, 1973), while in the dog femoral vein ATP and AMP

concentrations are raised during sciatic nerve stimulation (Chen, Selleck & Scott, 1972). There is no evidence supporting the view that the release of adenyl compounds under these conditions causes pain. An algogenic action is much more likely to occur under conditions where removal of adenyl compounds from the site of release is impaired, as is the case in ischaemia, or when there is extensive cellular damage. Sustained or repetitive muscle activity with intact circulation produces tiredness, dull, aching pain and stiffness (Barcroft, Foley & McSwiney, 1970). With the circulation occluded, ischaemic pain is almost invariably experienced and under these conditions the concentration of ATP in plasma is raised above that in the absence of occlusion (Forrester, 1972). A raised level of adenosine has also been observed in the coronary sinus blood of dogs with experimentally induced cardiac ischaemia (Rubio, Berne & Katori, 1969).

High concentrations of ATP have previously been reported to produce pain in man when applied to the human blister base (Keele & Armstrong, 1964) or injected intradermally (Stoner & Green, 1945). In mice, Collier, James & Schneider (1966) found that ATP administered intraperitoneally induced abdominal writhing which is interpreted as being a nociceptive response. There are no reports of an algogenic action of ADP, AMP or adenosine. The degradation products of adenine nucleotides, inosine and its monophosphate derivative and hypoxanthine are devoid of an algogenic action (unpublished results).

For the identification of the adenyl compounds as the algogenic material the rabbit isolated jejunum was particularly useful since it gave biological responses (relaxations) which showed a parallelism with pain responses. This also applied to certain test samples in the case of the rat isolated stomach strip preparation which responded with contractions. The main difference between the two isolated organ preparations and the human blister base was that whereas on the latter the three nucleotides had comparable potencies, on the two former the order of potency was ATP > ADP > AMP with AMP having least activity on the rat isolated stomach strip preparation.

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