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# THE EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON PLATELET AGGREGATION AND PROTEIN SYNTHESIS

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**Abstract**—This study investigated the role of platelet protein synthesis in platelet aggregation. Cycloheximide (Cx) and chloroamphenicol (Cm) were used as inhibitors of cytoplasmic (80S ribosome directed) and mitochondrial protein synthesis respectively. The effect of these agents on human platelet aggregation and L-[U-$^{14}$C]leucine incorporation into platelet protein was investigated.

Cm exhibited dose-dependent inhibition of collagen, thrombin and secondary ADP aggregatory responses, but had no effect on arachidonate or primary ADP responses over a similar concentration range ($3.1 \times 10^{-3}$, $3.1 \times 10^{-4}$ and $3.1 \times 10^{-5}$ M). Cm also inhibited platelet secretion associated with collagen and secondary ADP responses. Furthermore, Cm exhibited a similar dose-dependent inhibition of L-[U-$^{14}$C]leucine incorporation into platelet protein reaching 80% inhibition of incorporation at $3 \times 10^{-3}$ M.

At similar concentrations ($3.5 \times 10^{-3}$, $3.5 \times 10^{-4}$ and $3.5 \times 10^{-5}$ M) Cx failed to show inhibition of human platelet aggregation by all agonists used with the exception of collagen where some inhibition was seen at high Cx concentration ($3.5 \times 10^{-3}$ M). Cx was also found to be ineffective at inhibiting L-[U-$^{14}$C]leucine incorporation into platelet protein at all concentrations tested.

These results suggest that the majority of platelet protein synthesis is mitochondrial and that this protein synthesis may have a role in human platelet aggregation.

Protein synthesis in platelets was discovered simultaneously by Booyse and Rafelson [1, 2, 7] and Warshaw *et al.* [3, 4] who observed that puromycin could block the incorporation of L-[U-$^{14}$C]leucine into platelet trichloroacetic acid precipitable material. Puromycin is a structural analogue of the amino-acyl adenosine moiety of tRNA and causes the premature termination of elongating polypeptide chains on all ribosomal types. Booyse and Rafelson suggested that the main product of this protein synthesis was the platelet contractile protein, thrombosthenin. It was further suggested that since platelets are anucleate and no DNA or RNA synthesis was detectable within them, long-lived mRNA derived from the megakaryocyte precursor cell could be responsible for directing the protein synthesis.

This evidence, however, contrasts with that of Agam *et al.* [5] who observed both RNA and DNA turn-over in platelets and associated both with the platelet mitochondrial system. Also Battacharyya *et al.* [7] have observed organellar linked thymidine kinase activity. These findings suggest that at least part of the protein synthesis observed by Warshaw *et al.* and Booyse and Rafelson may have been mitochondrial.

Since puromycin inhibits protein translation on all ribosome types it was impossible for either group to determine which fraction of protein synthesis was cytoplasmic or mitochondrial and which might be important in platelet physiology. Neither Booyse and Rafelson nor Warshaw *et al.* studied the effect of inhibition of platelet protein synthesis on aggregation.

It is interesting to note that amongst many antibiotics that have been studied for their effects on platelet aggregation Genua *et al.* [8] and Murer and Siojo [9] have observed that chlortetracycline is particularly effective at inhibiting platelet aggregation dependent upon secretion of ADP. Murer and Siojo suggested that this was as a result of chlortetracyclines ability to complex with $Ca^{2+}$ disturbing the intracellular flux of the ion, an important intracellular messenger in platelet aggregation. This observation is also interesting because chlorotetracycline has another function in that it is an inhibitor of mitochondrial protein synthesis.

This study uses a specific inhibitor of both 80S ribosome directed protein translation, Cx [10], and 70S ribosome and related types, e.g. mitoribosome directed protein translation, Cm [11], to attempt to study in greater detail protein synthesis within the human platelet and its possible importance in platelet aggregation. (The effects of these drugs on mitochondria are reviewed in [12]).

## MATERIALS AND METHODS

*Chemicals and radiochemicals.* L-[U-$^{14}$C]leucine was obtained from Amersham International plc (Amersham, U.K.) (348m Ci/mM aqueous solution, 50 $\mu$Ci/ml), Cm, Cx, ADP, thrombin, sodium arachidonate, prostaglandin $I_2$ ($PGI_2$), apryase and hiru-

din were also obtained from the Sigma Chemical Co. (MO, U.S.A.) and collagen from Hormon-Chemie (Munchen, F.R.G.). Cm and Cx solutions were made up in methanol to 200 mg/ml, filter sterilized and diluted with methanol as required. All the agonists were used as solutions: ADP in physiological saline (1 mg/ml), sodium arachidonate in 0.1 m $NaCO_3$ ($O_3$ free) (25 mg/ml), thrombin in physiological saline (250 U/ml) and collagen in isotonic glucose pH 6.7 (1 mg/ml). Stock solutions were diluted with the appropriate solvent as required. All other chemicals were standard laboratory grade reagents supplied by either BDH Chemicals Ltd. (Dorset, England) or Sigma Chemical Co. (MO, U.S.A.).

*Isolation of human platelet-rich plasma (PRP) for aggregation studies.* Platelets were obtained immediately before needed from volunteers who had denied taking medication for at least two weeks prior to the study. Blood taken from the antecubital vein was immediately anti-coagulated with 3.8% w/v trisodium citrate containing 0.078 M D-glucose adjusted to pH 6.5 with NaOH. The whole blood was centrifuged at room temperature for 20 min at 800 rpm (250 *g*) in a MSE bench centrifuge. PRP was separated from red blood cells and used within 2 hr of vein puncture.

*Isolation of washed human platelets for* L-[U-$^{14}$C]*leucine incorporation studies.* Human PRP was isolated as described above except that Aster-Jandls ACD [13] $\frac{1}{6}$ volume was used as anticoagulant. Prostacyclin (1 μg/ml), apyrase (1 μg/ml) and hirudin (0.1 U/ml) were added to the PRP prior to centrifugation at 450 *g* (22°) for 25 min (MSE bench centrifuge). The resulting pellet was then gently resuspended in calcium-free Tyrodes solution and the platelet count adjusted to $2 \times 10^8$ pl/ml. Platelet counts were determined using a Coulter counter Z-1B and PRP suspensions checked for leukocyte and erythrocyte contamination which was always less than 1 cell/4.500 platelets.

*Measurement of platelet aggregation.* Platelet aggregometry was essentially carried out as described by Butler *et al.* [14] using a dual channel optical aggregometer (Payton Ass. Inc., NY). Compounds of interest were co-incubated with PRP (37°) in capped plastic vials for an appropriate time prior to aggregation studies. Test compounds never exceeded 0.5% v/v of incubation volume, and controls, treated with an appropriate volume of solvent alone, were performed. Dose–response curves were obtained to a range of agonists using rate of aggregation for quantitation. Platelet secretion was measured indirectly using a Payton Lumiaggregometer (Payton Ass. Inc., NY) as described by Ambler and Wallis [15].

*Measurement* L-[U-$^{14}$C]*leucine incorporation into platelet protein.* A suspension of washed, titred human platelets was divided into 1.0 ml aliquots and incubated in 25 ml disposable plastic centrifuge tubes (Sarsted 250 No. N-55-46-8 polypropylene) on a heater block at 37° in the presence of 1.4 μmoles L-[U-$^{14}$C]leucine (10 μl of 50 μCi/ml stock solution) for increasing periods of time. One series of such tubes contained a compound of interest at constant concentration and another served as a control, containing an appropriate volume of solvent alone. At known time intervals a control and treated sample tube were removed from the heater block and 2.5 ml of hot (90°) 20% trichloroacetic acid (TCA), 10 mM L-leucine added. The tubes were then heated at 90° for 20 min. The resulting precipitate was pelleted by centrifugation and resuspended by aggitation in 5 ml of 10% TCA, 5 mM L-leucine. This washing procedure was carried out three more times and was followed by one wash of the precipitate in a 1:1 v/v mixture of ethanol and ether and one wash in ether alone. Finally the precipitate was pelleted and allowed to dissolve overnight at 45° in 1 ml of soluene 350 (Packard Instruments Co. Inc., IL). 4 ml of luma gel scintillation fluid (Packard Instruments Co. Inc., IL) was added to the dissolved precipitate and samples were counted for 1 hr each in a Rack Beta scintillation counter (LKB Instruments Ltd., Surrey, England). A quench correction was applied to counts and cpm have been expressed as μmoles of L-[U-$^{14}$C]leucine.

## RESULTS

The results indicated that Cm was effective at inhibiting the aggregation *in vitro* of human PRP induced with collagen (Fig. 1). All concentrations of Cm tested raised the threshold concentration of collagen required to aggregate the PRP from 0.1 to 0.3 μg/ml, an increase of 3 times. Cm's effect on platelet aggregation was dose-dependent in that increasing concentrations of Cm led to increasing degrees of inhibition of platelet aggregation. All concentrations of Cm studied shifted the dose–response curve to the right and the highest concentration, $3.1 \times 10^{-3}$ M reduced the maximum possible rate of aggregation. Cx at similar concentrations, $3.5 \times 10^{-3}$, $3.5 \times 10^{-4}$ and $3.5 \times 10^{-5}$ M had little observable effect on the aggregation *in vitro* of human PRP induced with collagen. At the highest concentration used, $3.5 \times 10^{-3}$ M, Cx may have marginally inhibited platelet aggregation. The effect of $3.1 \times 10^{-3}$ M Cm on the aggregation of

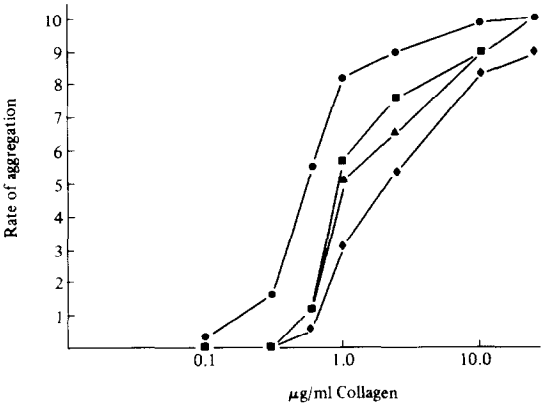


Fig. 1. The effect of Cm on aggregation, *in vitro*, of human PRP by collagen. Human PRP preincubated for 2 hr at 37° with (a) $3.1 \times 10^{-5}$ M Cm (■), (b) $3.1 \times 10^{-4}$ M, Cm (▲), (c) $3.1 \times 10^{-3}$ M Cm (◆), and (d) solvent alone (as a control) (●), was induced to aggregate with collagen as described previously. This result is typical of a number of identical experiments.

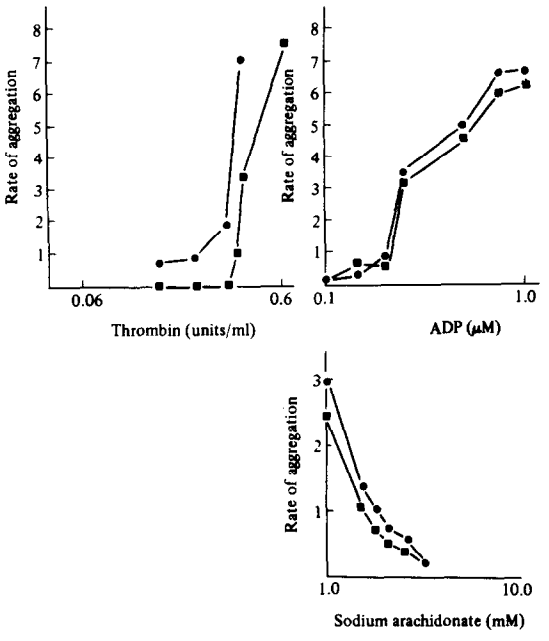


Fig. 2. The effect of $3.1 \times 10^{-3}$ M Cm on aggregation, *in vitro*, of human PRP by: thrombin, ADP and sodium arachidonate. Human PRP preincubated for 2 hr at 37° with $3.1 \times 10^{-3}$ M Cm (■) and solvent alone (as a control), (●) was induced to aggregate with (a) thrombin, (b) ADP, and (c) sodium arachindonate as described previously. These results are typical of a number of identical experiments.

human platelets by other agonists: thrombin, ADP and arachidonate is illustrated in Fig. 2. These results indicate that Cm is marginally effective at inhibiting aggregation produced in response to thrombin but is not inhibitory to aggregation when either ADP or arachidonate are the agonists. This work suggested that an effect on platelet secretion may have been responsible for Cm's effect on platelet aggregation. Subsequently, Cm's effect on secondary phase aggregation induced by ADP was studied, this phase is linked to secretion. Figure 3 illustrates that whilst $3.1 \times 10^{-3}$ M Cm had little or no effect on primary phase aggregation with ADP it was inhibitory to the secondary phase. Figure 3 represents the optical density changes of human PRP which had been pretreated with $3 \times 1 \times 10^{-3}$ M Cm for 2 hr and aggregated with ADP. The secondary phase aggregation is marked (Y). To further define Cm's effect on platelet secretion lumiaggregometry was used and Fig. 4 presents the results obtained for human platelet aggregation and secretion, when platelets had been pretreated with $3.1 \times 10^{-3}$ M Cm and aggregated with collagen. It is clear that at this concentration Cm is inhibitory both to platelet secretion and aggregation. Cm reduces the total possible amount of secretion and increases the threshold agonist concentration at which secretion is first observed. There is a close relationship between the inhibition of platelet aggregation and secretion. When $3.5 \times 10^{-3}$ M Cx was tested for an effect on the aggregation *in vitro* of human PRP by ADP, arachidonate and thrombin it was not observed to have any activity.

Having established that Cm was inhibitory to platelet aggregation its effect, along with that of Cx, on platelet protein synthesis was studied. Figure 5 contains the results of experiment in which washed, titered suspensions of human platelets were pretreated with increasing concentrations of Cm, incubated at 37° for various lengths of time with L-[U-$^{14}$C]leucine and monitored for uptake into platelet

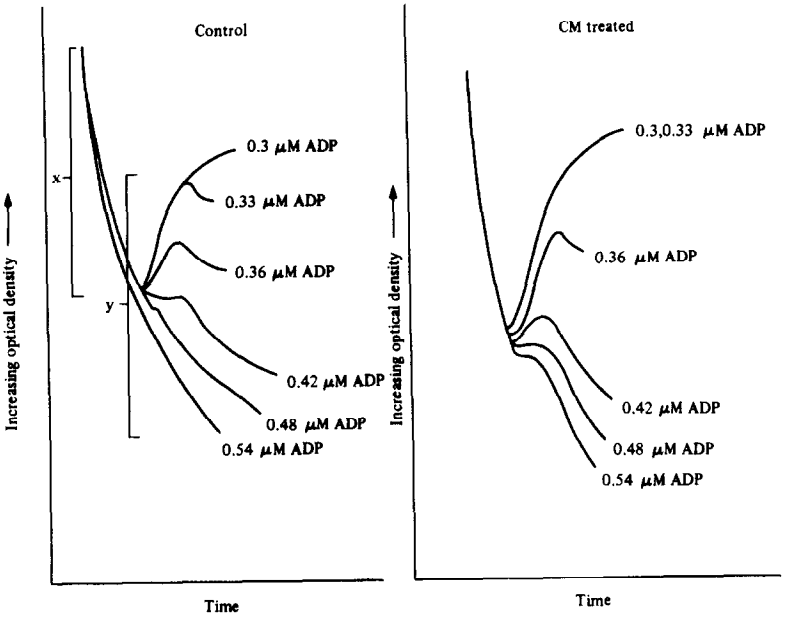


Fig. 3. The effect of $3.1 \times 10^{-3}$ M Cm on the aggregation, *in vitro*, of human PRP by ADP. Human PRP was preincubated for 2 hr at 37° with $3.1 \times 10^{-3}$ M Cm, and solvent alone (as a control) and induced to aggregate with ADP. The results are presented as recordings from a Payton dual channel aggregometer measuring change in optical density of the PRP with time. X marks the primary phase response of human PRP to ADP and Y the secondary, secretion linked response.

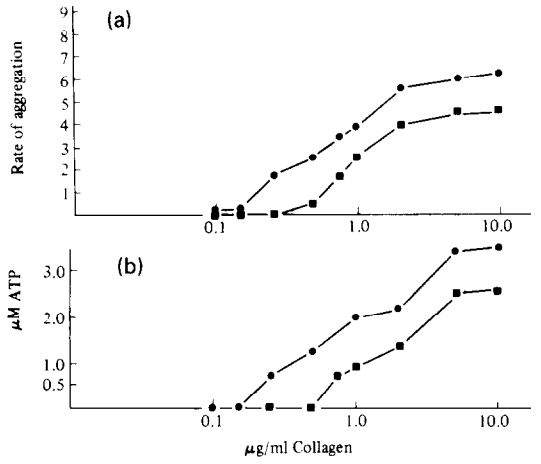


Fig. 4. The simultaneous effect of $3.1 \times 10^{-3}$ M Cm on aggregation and secretion, *in vitro*, of human PRP treated with collagen. Human PRP preincubated for 2 hr at 37° with $3.1 \times 10^{-3}$ M Cm, (■) and solvent alone (as a control), (●) was induced to aggregate with collagen in a Payton Lumiaggregometer using the method of Ambler and Wallis [14]. These results illustrate the comparative rates of (a) aggregation and (b) secretion of ATP of the PRP and are typical of a number of identical experiments.

protein. With increasing Cm concentration the amount preceded by additional matter of L-[U-$^{14}$C]leucine incorporated into platelet protein decreased. $3.1 \times 10^{-3}$ M Cm produced approximately 80% inhibition of L-[U-$^{14}$C]leucine incorporation. The effect of Cm on protein synthesis was similar in its dose-dependency to its observed effects on platelet aggregation. When $3.5 \times 10^{-3}$ M Cx was tested for its ability to inhibit platelet protein

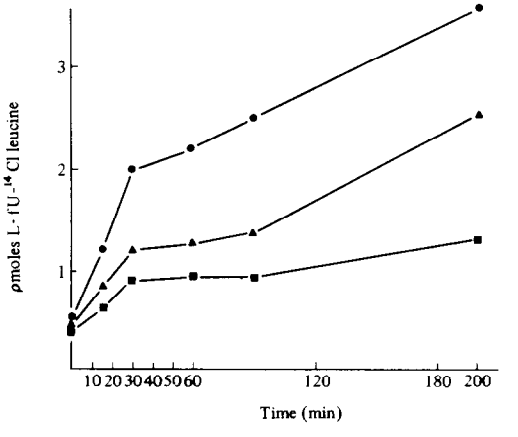


Fig. 5. The effect of $3.1 \times 10^{-3}$ M Cm on the incorporation of L-[U-$^{14}$C]leucine into human platelet protein. Suspensions of washed human platelets isolated in the manner previously described (Materials and Methods) with a density of $2 \times 10^6$ platelets/ml were incubated with (a) solvent alone (as a control) (●), (b) $3.1 \times 10^{-4}$ M Cm, (▲), and (c) $3.1 \times 10^{-3}$ M CM, (■), and L-[U-$^{14}$C]leucine. The suspensions were assayed at 0, 15, 30, 60, 90 and 200 min for incorporation of L-[U-$^{14}$C]leucine (see Materials and Methods). These results are typical of an identical series of experiments.

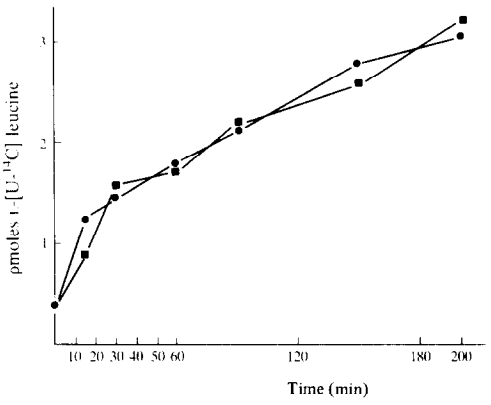


Fig. 6. The effect of $3.5 \times 10^{-3}$ M Cx on the incorporation of L-[U-$^{14}$C]leucine into human platelet protein. The experiment procedure was identical to that described in Fig. 5 with the exception that $3.5 \times 10^{-3}$ M Cx, (■) replaced $3.1 \times 10^{-4}$ M Cm and $3.1 \times 10^{-3}$ M Cm. The result is typical of a number of identical experiments.

synthesis no effect was observed (Fig. 6). To confirm that incorporation of the L-[U-$^{14}$C]leucine had been into platelet protein and not some other material a pronase digestion of a series of radioactive precipitates from one of the incorporation experiments was carried out. After digestion of the precipitate for 6 hr at 37° with 1 mg/ml pronase the radioactivity in the remaining precipitate and supernatant fractions was monitored. Digestion with pronase caused the release of >90% of the radioactivity from the precipitate into the supernatant in every case studied. This strongly supported the view that the L-[U-$^{14}$C]leucine was incorporated into platelet protein.

## DISCUSSION

The results of this study indicate that Cm was inhibitory to platelet aggregation by collagen in dose-dependent fashion with a maximum effect at the highest concentration tested, $3.1 \times 10^{-3}$ M. Cm was also marginally inhibitory to platelet aggregation by thrombin but had no observable effect on platelet aggregation induced by arachidonate. $3.1 \times 10^{-3}$ M Cm was able to disturb the secondary phase of platelet aggregation induced by ADP, which is secretion-linked, and could also lower the secretion response of platelets challenged with collagen. At the concentration used in this study Cx had little or no effect on platelet aggregation, by any of the agonists used. This evidence strongly suggests that Cm's inhibitory effect on platelet aggregation are linked to an effect of the drug on platelet secretion and that its little or no effect on aggregation by thrombin and arachidonate reflects the fact that aggregation can be brought about by mechanisms independent of secretion.

Our studies have also shown that a specific inhibitor of mitochondrial protein synthesis, Cm, can inhibit platelet protein synthesis whilst an inhibitor of 80S ribosome directed protein synthesis, Cx, does not, at the concentrations tested, have the same effect. Cm's effect on protein synthesis is similar

to its effect on platelet aggregation in that it is dose-dependent and $3.1 \times 10^{-3}$ M Cm produces the greatest effect. At this concentration Cm produces approximately 80% inhibition of protein synthesis, implying that the majority of protein synthesis in platelets may be organellar in origin. This evidence contrasts directly with that of Booyse and Rafelson who stated that the incorporation of L-[U-$^{14}$C]leucine observed in their studies was into platelet contractile protein. Our data do agree well, however, in a quantitative way with that of Warshaw *et al.* and platelet mitochondrial protein synthesis would appear to be possible in view of the findings of Agam *et al.* The dose correlation between the inhibition of protein synthesis and aggregation is intriguing and may indicate a need for organeller protein synthesis to maintain normal platelet physiology.

There is convincing evidence to associate mitochondria with a number of important platelet physiological processes. A supply of metabolic ATP from both glycolysis and oxidative phosphorylation appears to be required for platelet adhesion, clot retraction, secretion and aggregation [16] as well as platelet protein syntheis. Also mitochondria can sequester $Ca^{2+}$ [16] and although perhaps not of primary importance in resting platelet $Ca^{2+}$ metabolism may, as Scharf and Luscher [18] suggested, "mop up" excess intracellular $Ca^{2+}$ during platelet activation so that levels do not become inhibitory to cellular processes. Finally it has been shown that platelets are capable of synthesizing glycogen [19, 20] which is their principal energy reserve. Glycogen synthesis can depend upon a supply of Krebs cycle intermediates from the mitochondrion, which are corrected via phosphoenol pyruvate and glucose-6-phosphate to glycogen [21]. It is plausible to suggest that mitochondrial protein turnover may occur to maintain mitochondrial integrity to perform these tasks.

It is also interesting to note that erythromycin, a macrolide antibiotic capable of inhibiting protein synthesis by yeast but not mammalian mitochondria [22] did not have any effect on either platelet aggregation or protein synthesis in some preliminary experiments we have performed.

If mitochondrial protein turnover is important in certain aspects of platelet aggregation it is certainly worthy of further study as it may (a) help us to understand more clearly the molecular mechanisms involved in platelet aggregation and (b) indicate a new route for the treatment of certain platelet disorders.

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