An Upper Limit for Acetylcholine Content and Synthesis in Human Erythrocytes.* (21012)

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This study was made as a result of a revival of interest in the possibility of acetylcholine† (ACh) synthesis in mammalian red cells. Physostigmine disturbs the electrolyte transport process in erythrocytes and the poor stability of canine cells in isotonic KCl is improved by adding ACh(1-3). It is well-known that the synthesis and breakdown of the ester are intimately linked with excitability in several tissues and thus inferentially with cation movements(4,5), but the basic mechanism of the maintenance of stable concentrations of cations in red cells remains a matter of speculation. If ACh metabolism is related to cation movements in the red cell, it should be possible to show that the order of magnitude of ACh turnover is large enough to furnish the minimum free energy required for the accumulation of K ion against a concentration gradient. The order of magnitude of ACh turnover may be estimated from the data presented in this communication.

Methods. Blood was drawn in the laboratory from several apparently healthy donors.‡ The samples were either lightly heparinized or defibrinated immediately, and spun in the cold. In some experiments, physostigmine was added to the blood immediately after it was drawn, with no demonstrable effect on the ACh content. Samples were usually taken in plasma or serum but washing the cells with saline did not appear to alter the results. Hemolyzates were prepared by rapid freezing and thawing of the packed cells. Samples of whole blood or cells in saline were usually 10 ml in volume and were precipitated with 5 ml of 20% TCA, yielding approximately 10 ml of extract of cell-saline suspensions or 6 ml of whole blood extract. TCA was removed by ether extraction. In some experiments, K was removed by ion exchange(6), the sample being poured through a column of IRC-50 ion-exchange resin, 1 cm in diameter and 2 cm long, initially in the Na form. Following this, elution was begun with 0.85% saline until a total of 30 ml had been collected. Since the bath volume on the muscle was 7.5 ml 4 samples were available for direct application to the test preparation and for the preparation of the usual blanks and calibration standards containing known amounts of added ACh. In testing for *choline acetylase activity*,§ red cells obtained from heparinized or defibrinated blood were treated with 10 volumes of analytical-grade acetone at –13°C for 20 minutes. This procedure was repeated and the suspension filtered, washed with acetone, and dried briefly under vacuum at 2°C. The product, a fine, brick-red powder, was stored over a desiccant at –20°C. Powders were used within 2 to 24 hours after preparation, with a few exceptions. Rat brain powders were prepared by a method similar to that suggested by Feldberg(7). Acetone-dried powders were suspended in ice-cold saline containing 0.17 g % L-cysteine. The usual concentrations of powders were: red cells, 80 mg/ml; rat brain, 15 mg/ml. Suspensions were homogenized in a Potter-Elvehjem homogenizer for 2 minutes at 15-minute intervals for 1 hour. The homogenates were spun and the supernatants were used without further treatment. Preparations were kept cold at all times. *Acetylation tests* were run in Thunberg tubes at 38°C for 1 hour. Incubates, usually 5.5 ml, contained 3 ml of

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† Abbreviations are as follows: ACh, acetylcholine; TCA, trichloroacetic acid; ATP, adenosinetriphosphate; CoA, coenzyme A; DFP, diisopropyl fluorophosphate.

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extract and included in final concentration 6 mg of KCl, 3-6 mg of choline chloride, 4 mg of $MgCl_2 \cdot 6H_2O$, 15-30 mg of sodium citrate, 1.5-6.8 mg of sodium acetate, 2 mg of NaF, 5.4 mg of $Na_2HPO_4 \cdot 7H_2O$, 0.6 mg of physostigmine salicylate or 0.2-0.9 mg DFP, 4-8 mg of Pabst sodium ATP, usually both 0.4 mg of Pabst CoA and 0.3-0.4 ml of boiled yeast juice (200 mg of yeast/ml) and 0.85% NaCl to make the volume to 5.5 ml. In some experiments, either the CoA or the yeast was omitted. Normally, the ratio of K^+ to Na^+ was approximately ¼ but, in some experiments, K^+ was substituted for Na^+. Three experiments at an approximate K^+/Na^+ ratio of 4, one at 1/1 and 3 at ¾ were conducted. Syntheses were conducted under an atmosphere of 95% N_2-5% CO_2 and, at their conclusion, the incubates were inactivated with TCA or HCl, and centrifuged. In some cases, TCA was removed with ether but in others samples were neutralized with NaOH since only small amounts of protein precipitant were required. Various control syntheses included incubation with ACh to determine any systematic loss, and incubation in the absence of any one or a combination of the following: choline, acetate, citrate, CoA, physostigmine. Yields are reported as the amount in micrograms synthesized during one hour of incubation per gram of dry powder (γ/g/hour). The *physostigmine-sensitized* frog rectus abdominis used by Chang and Gaddum(8) and the acetone-treated rectus abdominis method suggested by Chang *et al.*(9) were used for the biological estimation of ACh. Our best results‖ were obtained with *Rana pipiens* and, on the whole, results with *R. clamitans* were inferior. Frogs were kept successfully for considerable periods in aquariums containing about 1 inch of water at 10°C. Large, egg-carrying females often seemed particularly satisfactory. Contractions of the rectus abdominis were recorded electrically on a strip paper chart(10). When physostigmine potentiation was to be used, the dissected muscles were stored in amphibian Locke solution in the cold for about 18 hours prior to assay, with generally improved results. On several occasions, muscles retained adequate sensitivity for as much as 5 days. Estimations in the range of 0.15-0.5 γ of ACh in a 7.5-ml bath were made by this method. For assays in which the acetone-treated rectus was used, muscles were dissected immediately before use. Sensitivities were in the range of 0.025-0.25 γ in a 7.5-ml bath containing 0.05-0.2 ml of acetone.

Results. The presence of ACh in TCA extracts of treated and untreated cells could not be demonstrated by the physostigmine-sensitized rectus. Cells were incubated at 25° and 38°C for 1 to 9 hours in the presence and in the absence of the cholinesterase inhibitors physostigmine, 1×10^{-4} to 8×10^{-3} M, and DFP, 5×10^{-6} to 1×10^{-3} M. Esterase-blocked cells were equilibrated in their own plasma and in suspensions of saline with and without added dextrose. Accumulation of ACh in suspensions of cholinesterase-blocked cells and hemolyzates at physiological temperature could not be detected aerobically or anaerobically. The addition of choline, acetate, and citrate did not alter the result. In some cases, ATP-ase was blocked with fluoride to preserve the normal ATP of the cells. Hemolyzates incubated anaerobically with the complete synthetic system used in the tests with acetone powder extracts synthesized less than 0.3 γ of ACh per ml of hemolyzate. The limit of detection in the remainder of the experiments varied between 0.26 and 0.09 γ of ACh per ml of packed cells. ACh was not detected in this range in any of the experiments. The complete recovery of added ACh in quantities of 0.1-0.4 γ per ml of cells assured no systematic loss.

Additional experiments in which slightly higher sensitivity of detection was achieved by acetone potentiation indicated that red cells contain less than 0.08 γ of ACh per ml. No detectable increase could be observed by incubation for 2 hours in the presence of esterase inhibitor. Small contractions occurred in some instances, but they were either unaltered or were increased by alkaline hydrolysis. They were thus not caused by ACh.

The estimated upper limit of ACh synthesis

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by acetone powders of red cells was compared with syntheses by rat brain controls. It is clear from the discussion of *methods* that syntheses were conducted under a variety of experimental conditions, *i.e.*, the age of the red cell powders (prepared from the blood of several donors) was varied; either DFP or physostigmine was used to block cholinesterase; either CoA or yeast juice was frequently omitted; the ATP concentration was varied; either acetate or citrate or both were used as substrates. The results were unaffected by any of these modifications with the exception of the last. Brain extracts synthesized between 430 and 1040 γ ACh/g/hr if either acetate or citrate was used as the substrate and under these conditions synthesis by red cell powders was below the threshold of detection (2.8-13.2 γ ACh/g/hr). The inclusion of brain activator(11) in one experiment did not affect the result. Small contractions were occasionally observed at this level but they were shown to be artifacts. Results of additional experiments showed that maximal brain syntheses (1200-1670 γ ACh/g/hr) were obtained if *both* acetate and citrate were used as substrates. Under these optimal conditions small amounts of biologically active material were frequently produced by extracts from red cell powders (1.1 or less to 6.3 or less γ ACh/g/hr). Inclusion of purified pigeon liver extract did not materially improve the synthesis(12). Extracts from a system in which the white cells had been concentrated showed no increased synthesis. Red cell synthesis was not reduced if the *dry* powder was heated in a boiling water bath for 1 hour, or if the powder was extracted without cysteine and the extract incubated with $CuSO_4$. Synthesis by blood-brain extracts treated similarly was reduced by 50%. Synthesis by brain alone was reduced by 80% if the powder was extracted without cysteine and the extract incubated with $CuSO_4$. In addition, synthesis by red cells was not altered if the *extract* was heated at boiling temperature for a short time but synthesis was markedly reduced if boiled extracts were redried with acetone, the powder obtained extracted with saline, and incubated as a typical extract. The effect of the synthesized material was reduced by boiling at alkaline pH but was not enhanced by treating the rectus with physostigmine, whereas extracts containing added ACh showed physostigmine potentiation. It was found that the activity was unaltered by atropine. No increase in biological activity was produced in syntheses at high K^+/Na^+ ratios. Syntheses were clearly less than 3 γ/g hour. Alkaline hydrolysis generally produced less reduction of the small biological effect, which was entirely the same with or without physostigmine treatment. Recovery of ACh added in amounts which correspond to synthetic rates of 3-100 γ/g/hr was quantitative in the experiments reported.

TABLE I. Effect of Red Cell Powder on the Rate of ACh Synthesis by Brain Preparations.
Brain powder extract 30 mg/ml, brain-blood powder extract 60 mg/ml concentrations in saline-cysteine solutions. Amount of extract shown as equivalent mg of powder extracted. Synthesis in γ/g/hr.

Brain		Brain-blood		
	ACh	Amount (mg)		ACh
Amount (mg)	synthesis (γ/g/hr)	Brain	Blood	synthesis (γ/g/hr)
97.3	1600	100.3	100.3	760
48.6	1300	49.4	148.2	770
24.3	1200	22.5	157.5	1600

Inhibition of brain extract activity occurred in several experiments in which red cell powders were added during extraction. This was not invariable, however, and in one series of experiments (Table I) where particular care was used to maintain uniformity of extraction procedure, the largest ratio of blood powder to brain powder during the extraction actually produced a greater synthesis than in the control. Synthesis by brain extract was not affected by a hemolyzed red cell preparation.

Acetone effects on the muscle. Results of a number of experiments indicated that contraction of the rectus in the presence of acetone may constitute a less specific test for ACh than that provided by the physostigmine-sensitized preparation. Investigation of some of the properties of acetone potentiation included determinations of its effect on the choline, K, ATP, and ACh response of the untreated, physostigmine-sensitized, and atropinized rectus.

We found that, in the presence of ace-

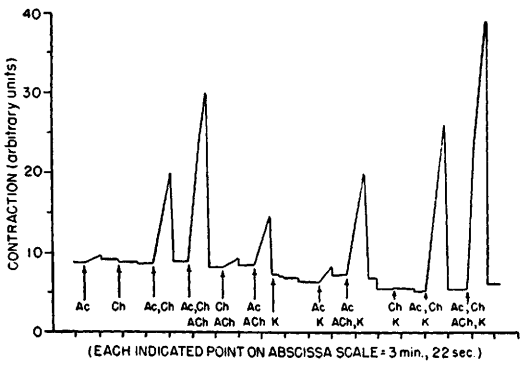

FIG. 1. The effect of choline, K, and ACh on the acetone-treated rectus. Each contraction recorded for 3 min. beginning at arrow (↑). Recorder off during 10-min. wash. Total bath volume, 7.5 ml including the following additions as noted in the figure: Ac = 0.1 ml of acetone, ACh = 0.1 γ of acetylcholine, Ch = 0.3 mg of choline chloride, K = 3 mg of KCl.

tone, the rectus was sensitized to choline and K as well as to ACh. Choline and K contractions resemble ACh contractions both in the shape of the curves described and in the rapidity of relaxation on removal of the stimulating agent. In addition, as with physostigmine potentiation, the ACh response of the acetone-treated rectus may be potentiated by choline and K in threshold and subthreshold concentrations. Fig. 1 illustrates some of the contractions which were recorded. In Fig. 2 it may be seen that physostigmine appears to *enhance* the effect of ACh on the acetone-treated rectus (contractions 1, 3, 5) and that atropine appears to counteract the effect of the physostigmine to a greater extent in the absence than in the presence of acetone (contractions 5 and 6, and 3 and 7), but that the *direct* action of ACh on the acetone-sensitized muscle is not depressed by atropine (contractions 7 and 1). It seems, then, that the acetone-treated rectus is subject to a number of effects which may simulate and/or potentiate the effects of ACh. The frog rectus may not serve as an adequate assay material in syntheses in which acetone powders of low activity are used without removing any acetone which the samples may contain. Threshold activity detected in these systems may be due to potentiation by residual acetone to ACh, choline, K, some other active substance, or to a combination of these substances. Loss of activity of the samples boiled at alkaline pH may mimic alkaline hydrolysis but may be caused by loss of acetone. The nearly equal response of the rectus to these samples in the presence or absence of physostigmine and atropine may be attributed to the presence of acetone. It is recognized that acetone potentiation can only partially account for the effects observed in our red cell experiments and that the results were probably also influenced by the presence of other unidentified substances.

Discussion. A number of reports in the literature attest to the low level of ACh in mammalian erythrocytes. Chang and Gaddum(8) showed that the level in dog, horse, and ox cells was 0.05-0.08 γ per ml of blood. The results suggested substances other than ACh may be responsible for biological activity detected at this level. Bülbring *et al.*(13) were unable to find ACh in rat cells. Synthesis by packed physostigmine-treated cells was undetectable. Quastel *et al.*(14) set the upper limit of synthesis in physostigmine-treated guinea pig blood at 0.3 γ per hour per 100 mg dry weight. Our results in man fully confirm

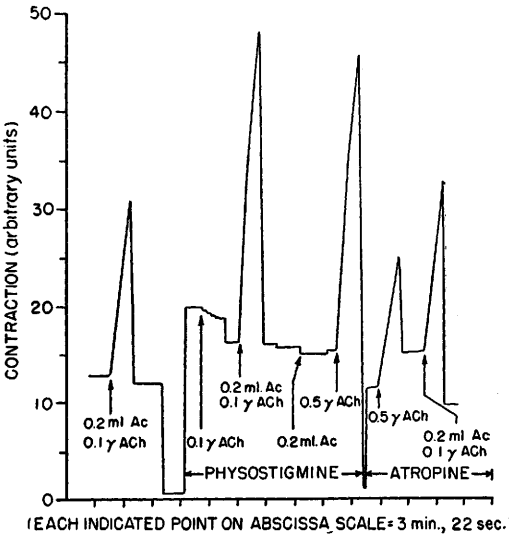

FIG. 2. The effect of acetone (Ac) on the ACh response of the untreated, physostigmine-sensitized, and atropinized rectus. The muscle was treated with 8×10^{-6} g/ml of physostigmine salicylate for 52 min. and atropinized with 6×10^{-7} g/ml of atropine sulfate for 20 min. Contractions recorded as in Fig. 1, bath volume as in Fig. 1. All additions to bath noted in figure.

the low level of erythrocyte ACh, and it would seem not unlikely that this will be generally true in mammalian red cells. The low value of synthesis is in accord with the low value of the observed choline acetylase activity but it could also be explained by postulating that there is at all times a small amount of bound ester which can be replaced by synthesis only when it is depleted. A simple calculation will show that if this were so and if all this material were uniformly spread on the surface of the erythrocyte each molecule would occupy approximately 5 x 10^{-3} square micra with a spacing of at least 700 Å, or approximately one percent of the diameter of the erythrocyte. Of course the possibility remains that ACh exists with closer spacing in only a portion of the cells.

The observed synthetic power of the cell powders is less than that which has been previously reported(15) and is exceedingly low compared to that found in tissues in which ACh is known to play a physiological role. When choline acetylase activity from an outside source is present most and sometimes all of it can be observed in red cell powders. Bülbring *et al.*(13) were able to observe synthesis of ACh by trypanosomes in the presence of red cell material. Although some inhibition by red cell powders is occasionally observed in our experiments it is never more than 50%. Even in the worst cases there is enough synthesis by brain enzyme in the presence of red cell material to indicate that inhibition alone can scarcely account for the low level of synthesis by red cells. Since red cell synthesis is not affected by treatment which reduces brain synthesis by 80%, it is possible that the activity observed is not the result of synthesis by choline acetylase. Nonenzymatic synthesis or synthesis by some other enzyme may occur. It is possible, of course, that choline acetylase activity exists in red cells but, in contrast to neural tissue, cannot be demonstrated by the standard procedures of extraction. A more likely interpretation is that the low level of synthesis reflects a general low level of acetylcholine metabolism in erythrocytes whose generally low metabolic activity is familiar.

The effects of physostigmine and DFP on ion transport in erythrocytes(1,2) suggest some relation between cholinesterase activity and ion movement. If the turnover of ACh could be verified and related in some way to the process of selective K accumulation in red cells, then the energy required could be conveniently related to the energy released in the hydrolysis of ACh. If the observed upper limit of choline acetylase activity in our experiments is accepted, the result is not encouraging for an ACh mechanism. Current estimates of the minimum free energy required to accumulate a K ion against the concentration gradient in human cells are about 1600 calories/mole(16). The hydrolysis of 1 mole of ACh yields not more than 3200 calories (17). Thus not more than two K ions can be accumulated per mole of ACh hydrolyzed if such a process is to account for the energy of selective K accumulation. Since about 2 x 10^{-6} equivalents of K exchange per hour in 1 ml of human cells(18) at least 10^{-6} moles of ACh would be synthesized per ml of cells or about 150 γ of ACh per ml. Since the ratio of the weight of packed cells to that of the acetone dried powder is about 3:1, this is at least 50 times as great as any synthesis which we have observed from powder extracts. Certainly synthesis of 3 γ/g/hr in physostigmine treated cells would have been detected. The result suggests that, even with a liberal allowance for inferior synthesis *in vitro* and other factors, ACh turnover can scarcely be involved in selective K accumulation in erythrocytes. This conclusion does not deny the possibility that cholinesterase in the envelope might play some role other than that involving the hydrolysis of ACh, but this remains to be elucidated.

Summary. The ACh content of human red cells is less than 0.08 γ/ml and no increase in synthesis can be observed in cells equilibrated with cholinesterase inhibitors. A synthetic rate higher than 6.3 γ/g/hr by acetone powders of red cells was not observed. There is some doubt that the activity observed was the result of enzymatic synthesis of ACh. Calculations show this rate to be less than 1/50 that required to provide the free energy essential for the accumulation of K against a concentration gradient. The metabolism of

red cell ACh as a mechanism by which the cells accumulate K is of doubtful importance.

1. Greig, M. E., and Holland, W. C., *Arch. Biochem.*, 1949, v23, 370.
2. Taylor, I. M., Weller, J. M., and Hastings, A. B., *Am. J. Physiol.*, 1952, v168, 658
3. Holland, W. C., and Greig, M. E., *ibid.*, 1950, v162, 610.
4. Feldberg, W., *Physiol. Rev.*, 1945, v25, 596.
5. Nachmansohn, D., *Biochim. et Biophys. Acta*, 1950, v4, 78.
6. Sheppard, C. W., Cohn, W. E., and Mathias, P. J., *Arch. Biochem.*, 1953, v47, 475.
7. Feldberg, W., *Methods in Medical Research*, 1950, v3, 95. The Year Book Publishers, Inc., Chicago.
8. Chang, H. C., and Gaddum, J. H., *J. Physiol.*, 1933, v79, 255.
9. Chang, H. C., Lin, T. M., and Lin, T. Y., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 129.
10. Davidson, J. B., *Science*, 1951, v114, 361.
11. Feldberg, W., and Mann, T., *J. Physiol.*, 1946, v104, 411.
12. Nachmansohn, D., Wilson, I. B., Korey, S. R., and Berman, R., *J. Biol. Chem.*, 1952, v195, 25.
13. Bülbring, E., Lourie, E. M., and Pardoe, U., *Brit. J. Pharmacol.*, 1949, v4, 290.
14. Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Biochem. J.*, 1936, v30, 1668.
15. Holland, W. C., and Greig, M. E., *Arch. Biochem.*, 1952, v39, 77.
16. Solomon, A. K., *J. Gen. Physiol.*, 1952, v36, 57.
17. Nachmansohn, D., and Wilson, I. B., *Advances in Enzymology*, 1951, v12, 259, Interscience Publishers, Inc., New York.
18. Sheppard, C. W., and Martin, W. R., *J. Gen. Physiol.*, 1950, v33, 703.

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Chylomicron and Clearing Reaction: Effect of Heat and of Refrigeration.* (21013)

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The *in vitro* reaction by which turbid fat suspensions may be cleared following the addition of plasma obtained from animals after the injection of heparin has been studied extensively by Anderson and Fawcett(1), Anfinsen *et al.*(2), and French *et al.*(3). The present study is concerned with the fat particles (chylomicra) themselves and with factors which alter their susceptibility to clearing.

Method. Samples of lipemic plasma were obtained from dogs approximately 3 hours after fat meals of 4-8 g/kg. Cream fat was fed except where indicated. The plasma samples were centrifuged (at 4°C for 30 minutes at 20,000 × G.) in a Servall angle centrifuge and the subnatant clear plasma removed. This was replaced by 0.85% NaCl, and the suspension was recentrifuged. This operation was repeated 3 times. The resulting saline suspension of washed chylomicra was used at once or stored at 4°C or −18°C and used within a few days. Chyle was obtained by cannulation of the thoracic duct of dogs 1-2 hours after feedings of 8 g of cream fat per kg, and washed suspensions of chylomicra from the chyle were prepared as described for the plasma. All clearing tests were performed as follows: 1 ml of chylomicron suspension (or lipemic plasma or diluted chyle) plus 4 ml 0.85% NaCl were warmed at 37°C for 10 minutes in a 19 mm Coleman cuvette. One ml of warm post-heparin plasma was then added, the time noted and the turbidity of the suspension determined immediately and at 10-minute intervals for one hour or longer. All determinations of optical density were made in a Coleman spectrophotometer at 650 mμ wave length. The tubes were incubated at 37°C in a water bath during the test. The samples of post-heparin plasma were obtained

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