of A-band width are only approximate. The relative change in A-band width compared to changes in the sarcomere width provide better measurements for comparison.

A plot of sarcomere width versus A-band width (see Fig. 7) shows that during the initial period of shortening (down to 60 percent reference length) relatively little change takes place in the width of the A-band. It is only after this stage that changes in the A-band width become exceptionally pronounced. These changes have been observed in rabbit psoas myofibrils and are considered to be the first stages in the formation of contraction bands. It was previously implied (*5*, *6*), however, that the formation of contraction bands was due to a thickening of the A-bands as they pushed up against the Z-membrane. Figures 3 and 4 indicate that there is an apparent separation of the A-band before it is actually pressed up against the Z-membranes. This is in disagreement with the hypothesis that contraction-band formation is due to a pushing of the A-band material against the Z-membrane. It seems probable from these photomicrographs that an actual separation of the material in the A-band does occur.

Szent-Györgyi and Holtzer (*7*) located specific materials within the sarcomere during immunological studies. Using antibodies specific for light Meromyosin (LMM) and heavy Meromyosin (HMM), they were able to separate the A-band material into two groups. The LMM appeared to be located at the lateral borders of the A-band whereas the HMM was concentrated in the center (M-band). They also observed A-band separation due to what they called migration of LMM toward the Z-membranes. The banding resulting from this migration formed "doublets".

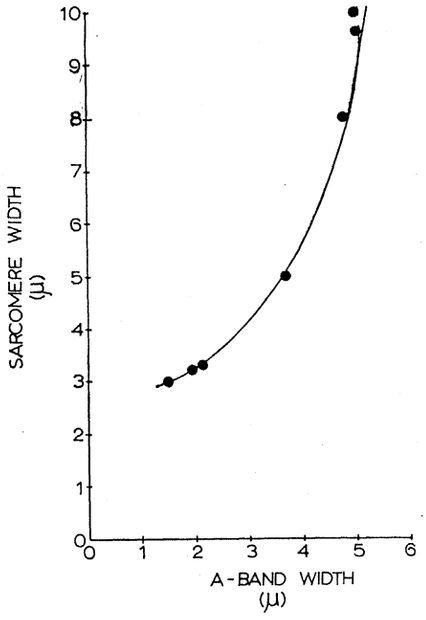

Fig. 7. The relationship between the length of the sarcomere and the width of the A-band in the barnacle myofibril. During the initial period of shortening (to approximately 60 percent of the reference length) relatively little change takes place in the A-band width.

Photographs by Hodge (*8*) support the hypothesis that A-band separation is a result of migration of the A-band material. The results reported here parallel exactly those reported by Hodge working with insect skeletal muscle.

The separation of the A-band into distinct areas of different materials provides a possible explanation for the origin of the observed contraction bands. Migration of the LMM region toward the Z-membranes would cause the observed A-band separation.

The problem of contraction in barnacle myofibrils has recently been considered by Hoyle and McAlear (*2*). They propose a situation whereby the A-filaments maintain a constant length. The observed changes, they maintain, are due to a "penetration" of the Z-disk by A-filaments. This is in disagreement with the findings presented here: Figs. 3, 4, and 5 show changes occurring in the A-band before it has come in contact with the Z-disk.

The separation of the A-band shown in Fig. 3 has been seen by a number of investigators in a variety of muscles (see *5*, *8*). The work of Hodge (*8*), in particular, shows that changes occur in the A-band when it is not in contact with the Z-disk.

If the contraction bands were formed through penetration of the Z-membranes by A-band filaments whose lengths did not change, then the minimum distance between the exterior edges of contraction bands (*X* in Fig. 6) would be equal to the A-band width (*A*). As shown by the supercontracted myofibril in Fig. 6, the distance is considerably less than the A-band width (*A*).

The results presented here support the hypothesis that muscular contraction depends upon the sliding of filaments, at least for contractions down to approximately 60 percent of the reference length. Contraction beyond 60 percent appears to be accompanied by a change in length of the A-filaments. These studies do not rule out the possibility of a shortened A-filament penetrating the Z-membrane.

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References and Notes

1. G. Hoyle and T. Smyth, *Science* **139**, 49 (1963).
2. G. Hoyle and J. McAlear, *ibid.* **141**, 712 (1963).
3. J. R. Bendall, *Biochem. J.* **81**, 520 (1961).
4. The composition of the solution was: 20 m*M* imidazole; 80 m*M* KCl; 5 m*M* potassium oxalate; 4.1*M* glycerol (after Bendall, *3*).
5. H. Huxley and J. Hanson, *Nature* **173**, 973 (1954).
6. H. Davson, *General Physiology* (Little, Brown, Boston, 1959).
7. A. G. Szent-Györgyi and H. Holtzer, *Biochim. Biophys. Acta* **41**, 14 (1960).
8. A. J. Hodge, *J. Biophys. Biochem. Cytol.* **2**, No. 4 Suppl., 131 (1956).
9. Supported by grant No. GM-09898, U.S. Public Health Service, and by the Life Insurance Medical Research Fund. The authors thank W. H. Johnson for his advice and encouragement during the course of this investigation.

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Cytochrome Content of Mitochondria Stripped of Inner Membrane Structure

Abstract. *The cytochrome composition of mitochondrial fractions which have been stripped of inner membrane subunits by exposure to high-frequency sound have been examined by low-temperature spectroscopy. The ratio of cytochrome* c *to cytochrome* a *is not changed by the treatment, but the concentration of cytochrome per milligram of protein is increased and the concentrations of cytochromes* c_1 *and* b *change slightly. These cytochromes* (c_1 *and* b) *may be at least partly located in the subunits of the inner membrane, but the idea that all the respiratory components are located in single subunits of the mitochondrial cristae may be considered to be disproved.*

Green and his co-workers propose as a plausible hypothesis that the inner membrane subunits of mitochondria (*1*, *2*) represent an integrated unit of all the components of the respiratory chain (*3*, *4*). However, some objections have been put forward, based upon what appear to be irreconcilible differences between the small size of the inner membrane subunits and the large size of the unit embracing the functions of electron transfer and oxi-

dative phosphorylation (the oxysome) (*5*). Additional evidence indicates that deficiencies of cytochrome *c* or of oxidase in the respiratory chain show no corresponding change in the size of the inner membrane subunits (*6*).

We report here the cytochrome content in membranes that have been stripped of inner membrane subunits by treatment with high-frequency sound. Low-temperature spectroscopy was used to ascertain the cytochrome content of the stripped membranes and the supernate fractions.

Mitochondria prepared from rat liver (*7*), were resuspended in 1m*M* phosphate instead of 0.25*M* sucrose and allowed to stand for 10 to 16 hours. The mitochondria were then treated with high-frequency sound at 1 amp for 40 seconds in the Branson instrument. After this treatment, three fractions were obtained by differential centrifugation for 10 minutes at 8000*g* and 40 minutes at 105,000*g*. These fractions were designated respectively, low-speed precipitate, high-speed precipitate, and high-speed supernatant.

Samples of all fractions were examined by low-temperature spectroscopy for cytochrome concentration. This technique (*8*) is of particular advantage in detecting and comparing amounts of cytochrome in materials whose fine structure has been observed in electron micrographs. The use of the method for detecting relative changes in the cytochrome is exemplified by Fig. 1, which shows the absorption bands of cytochromes *a*, *b*, c_1, and *c* in the low-speed fraction of rat liver mitochondria. The relative amounts of the cytochromes are computed from the ratios of the absorbancy changes divided by the appropriate ratios of extinction coefficients. Cytochrome content is also calculated on a protein basis, while the protein concentration is determined by the biuret method.

The relative changes in the cytochrome ratios in the various fractions are of principal interest in judging cytochrome content. Nevertheless, absolute concentrations of cytochromes can be determined, since it has been found experimentally that the intensification ratio under our particular conditions is 4.5 as determined by a comparison of room- and low-temperature difference spectra.

The sample is frozen in two different types of media, depending upon the requirements. When optimum reproducibility of the total absorbancy changes is desired, and when ease of manipulation is of importance, the samples are frozen in aqueous buffer (*8*). Where delineation of cytochrome c_1 is of importance, as is often the case, the 50 percent glycerol-water mixture is used (*8*). Succinate is added as a substrate and sulfide is added as a terminal respiratory inhibitor to reduce those components specifically concerned with electron transport. In the case of the high-speed supernatant fraction, dithionite is added also since cytochrome oxidase and presumably the reductases are absent from this material. The addition of succinate and sulfide is preferable in order to identify whether or not the intact electron-transfer system remains on mitochondrial membranes, particularly in the low-speed fraction.

The optical-density changes are measured as indicated in Fig. 1 against the baseline drawn in the figure. Cytochrome *a* is measured at 598 mμ, cytochrome c_1 at 553 mμ, cytochrome *c* at 548 mμ, and cytochrome *b* at 559 mμ. A baseline of the type illustrated in Fig. 1 can usually be obtained provided that the freezing has produced similar

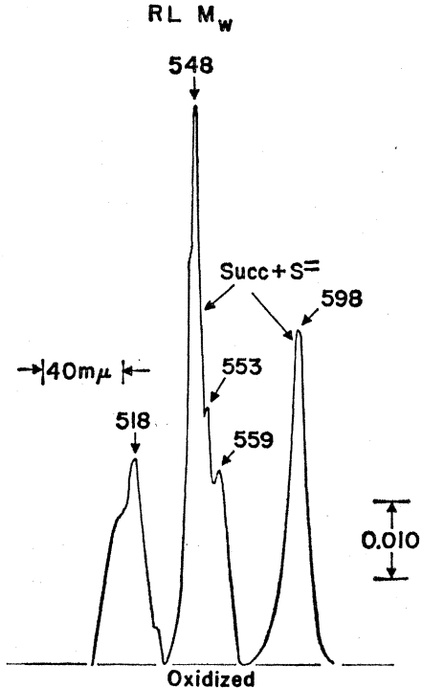

Fig. 1. Example of cytochrome assay by low-temperature (77°K) difference spectroscopy of rat liver mitochondria (Mw) suspended in 1m*M* phosphate buffer (*p*H 7.4) and allowed to stand for 10 hours. The bands of cytochromes *a* (+a_3), *b*, c_1, and *c* at 598, 559, 553 and 548 mμ are resolved.

Table 1. Analysis of fractions from 560 mg samples of rat liver mitochondria (Mw 1002) suspended in 0.25*M* sucrose.*

Total pr (mg)	Cytochrome *c* (mμ mole) Total	Per mg protein	Cytochrome ratios *a/c*	c_1/c	*b/c*
	M_w in inorganic phosphate				
560	115	0.21	0.70	0.58	0.52
	M_w in inorganic phosphate (after treatment with high-frequency sound)				
550	125	0.23	0.80	0.47	0.42
	Low-speed precipitate				
250	88	0.35	0.70	0.45	0.36
	High-speed precipitate				
58	17	0.29	0.63	0.46	0.61
	High-speed supernatant				
93	23+	0.25+	‡	‡	‡

* Our experiment Nos. 16 and 17. † Peak at 553 (cytochrome c_1). ‡ No cytochrome *c* present.

crystallinity in the two samples. In cases where the baseline is faulty and it is necessary to record the data, absorbancy differences may be measured with respect to appropriate reference wavelengths; 630 mμ for cytochrome *a*, 540 mμ for cytochrome c_1 and *c*, and 575 mμ for cytochrome *b*.

The low-temperature method is highly sensitive and requires only a small amount of material (0.6 ml of a 1μ*M* suspension of mitochondria is adequate for an oxidized-reduced determination). This is a matter of convenience in studying fractions obtained here, particularly in the high-speed supernatant fraction, in which the cytochrome concentration is rather small.

The isolated mitochondria were lysed by treatment for 10 to 30 minutes with a 1m*M* phosphate buffer, *p*H 7.4, if not previously so treated, and the sample was negatively stained with 2 percent potassium phosphotungstate, *p*H 6.8 by the surface-spreading technique (*1*). The dried preparations were examined in the Siemens Elmiskop I at 40,000 ×.

Electron micrographs show the results of treatment of rat liver mitochondria. This treatment lends, in the low-speed fraction, to a nearly complete stripping of the fine structure from the mitochondrial membranes. Figure 2, which is typical of this fraction, shows two major extrusions of the mitochondrial membrane of length roughly equal to 1 μ. On both sides of the two pieces of cristae, there appear to be less than ten subunits corresponding to an average spacing of approximately 3200 Å per subunit (total length of both surfaces of cristae is

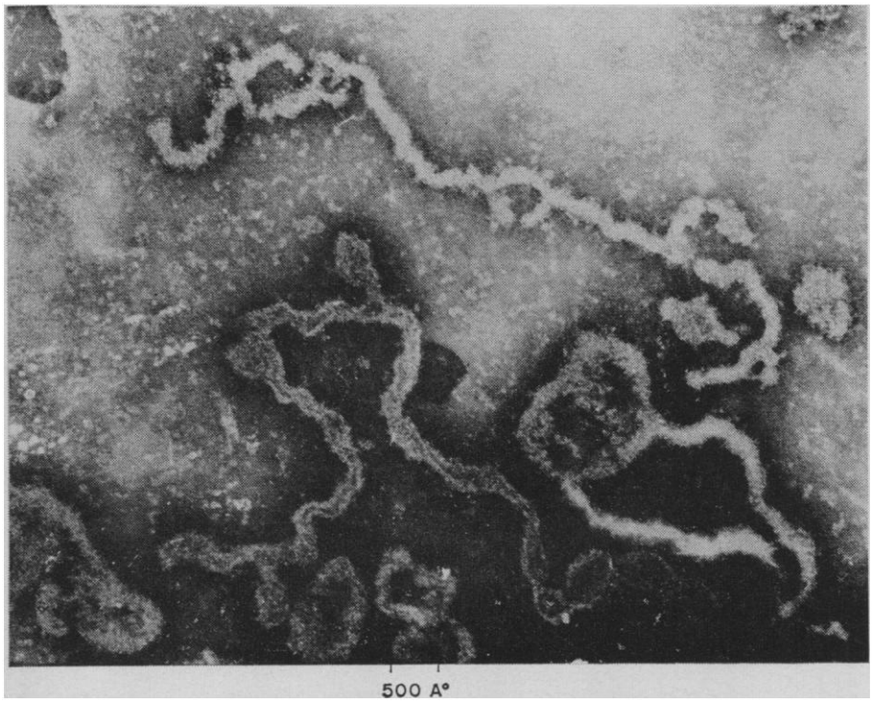

Fig. 2. Electron micrograph of extruded cristae of the low-speed precipitate fraction of mitochondria from rat liver (see Table 1). This may be compared with normal membranes (*1*).

32,000 Å, 32,000/10 = 3200 Å/subunit). This may be compared with the spacing of the subunits of approximately 100 Å observed in intact liver mitochondria (*6*). On this basis, the membranes are stripped to the extent of about 97 percent. However, the cristae of all mitochondria in the sample are not observable; approximately 80 percent of the mitochondria showed readily observable portions of extended crista. While it is justifiable to assume that the mitochondria in the electron microscope field which could not be clearly observed had the same degree of stripping as those that could be observed (97 percent), the most conservative estimate of the stripping is 80 percent. The possibility that inner membrane subunits that have been stripped from the cristae are bound elsewhere on the particles is unlikely since repeated washing of the low-speed precipitate fraction causes no significant change of cytochrome composition.

Table 1 shows a quantitative analysis of the fractions of rat liver mitochondria treated with phosphate and high-frequency sound. The first row indicates the recoveries of the total protein in the various fractions; 72 percent of the total protein is recovered, 45 percent of it being in the low-speed fraction, from which the electron micrograph of Fig. 2 is taken.

The second line indicates the recovery of cytochrome *c* and shows that the high-speed and low-speed fractions contain 90 percent of the cytochrome *c*. In addition, a cytochrome with a peak at 553 mμ was recovered in the high-speed supernatant fraction. This pigment is tentatively identified with cytochrome c_1, and by comparison with cytochrome c_1 in the mitochondrial fraction, it amounts to 34 percent of the total.

The concentration of cytochrome *c* on a protein basis is given in the third row, and there is actually more cytochrome *c* in relation to protein in the low-speed precipitate fraction than in the high-speed precipitate fraction. Thus, cytochrome *c* does not appear to have been lost from the particles.

Cytochromes *a*, c_1 and *b* are determined by the a/c, c_1/c, and b/c values (Table 1). The cytochrome *a* to *c* ratio is nearly constant in the various fractions, approximately 0.7. The recoveries of cytochromes c_1 and *b* in the low-speed precipitate fraction are also indicated in Table 1 by applying the c_1/b and b/c values to the total cytochrome *c* content in the various fractions. It is apparent that some c_1 and some *b* are lost from the respiratory chain after the first sound treatment even before fractionation. The amounts are, however, rather small. In addition to this loss, it is apparent that cytochrome *b* splits between the low-speed and high-speed fractions, being diminished in the low-speed fraction and increased in the high-speed fraction. Cytochrome c_1, on the other hand, is in the same relative concentration in these two fractions.

The data of Table 1, when considered in view of the electron micrographic studies, permit critical evaluation of the identification of the fine structure subunits (IMS) with cytochrome function. We have studied (*6*) biological cytochrome deficiencies in the flight muscle (cytochrome-*c* deficient) of the emergent bee and in the *Ascaris* worm (cytochrome oxidase deficient) and have found no correlation between the diameter of the inner membrane subunit and the cytochrome content. On the other hand, there was a correlation between the frequency of occurrence of the subunits and the amount of functional cytochrome in the mitochondria. As a result, we are now trying to evaluate critically what, if any, cytochromes may be identified with the inner membrane subunits. According to Table 1, the low-speed fraction which consists largely of bare membranes has an increased cytochrome content over that of the mitochondria themselves. The data seem to rule out any role for the inner membrane subunits in electron transfer, especially since we employed a "conservative" estimate of the cytochrome remaining in the IMS-free membrane. This was assayed as "functional" cytochrome by virtue of its reduction by succinate through electron transfer instead of by dithionite reduction. The ratios of cytochrome *a* to *c* indicate further that the relative amounts of these two components are retained in a largely IMS-free material. Changes are observed in cytochromes *b* and c_1 and one or both of these cytochrome components could be related to the inner membrane subunits. An increased concentration of cytochrome *b* is found in the high-speed particulate fraction. Although a small amount of a pigment resembling cytochrome c_1 is found in the supernatant fraction, there is only a small cytochrome c_1 deficiency in the low- and high-speed particle fractions.

Whether any electron-transfer component is associated with the subunits has yet to be proved; the demonstra-

tion of cytochrome reduction by substrate in the stripped membranes leads to the general conclusion that no essential component has been removed to the extent that electron transfer has been inhibited. The cytochrome assays rule out the presence of a_3, *a*, and *c* in the subunits, and the electron-transfer activity rules out DPNH and succinate dehydrogenases. However, our experimental method does not rule out oxidative phosphorylation cofactors or citric acid cycle dehydrogenases. The loss of some cytochromes *b* and c_1 from the stripped membrane is suggestive and needs further study. Thus, these experimental data appear to confirm our view (*4*, *5*) that the function of electron transport cannot be assigned to a single inner membrane subunit and confirms the possibility that the oxysome function of electron transport and oxidative phosphorylation involves a larger portion of the cristum than a single subunit and may extend along the cristum or into the cristum to meet the size requirements for the components of the oxysome (at least 170Å in diameter) (*5*). The possibility that other structural details may be identified with the oxysome function is suggested by the "dumbbell-like" structures of the cristum in flight muscle mitochondria (*9*).

Concerning the structure of the electron transport and oxidative phosphorylation components in the cristum, we find that the following hypotheses stand the test of continued experimentation. The respiratory chain consists of an assembly of cytochromes of approximately one each of the cytochrome (*10*) (particularly a_3, *a*, *b*, and c_1) with larger amounts of flavin NAD, quinone, and metal atoms; the respiratory components are intimately associated with the structure of the cristum, according to our early suggestions and those of Estabrook and Holowinsky (*11*) and Lehninger (*12*). Indeed, the idea of enzyme assemblies (*10*) has been extended to include a larger variety of enzymes of the cell, both in the citric acid cycle and in glycolysis under the term, "constant proportion enzymes" (*13*).

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References and Notes

1. D. F. Parsons, *Science* **140**, 985 (1963).
2. H. Fernandez-Moran, *Circulation* **26**, 1039 (1962).
3. D. Green, *Discovery*, Sept. 1962, p. 28.
4. D. Green, P. V. Blair, T. Oda, *Science* **140**, 382 (1963).
5. B. Chance, C. P. Lee, R. W. Estabrook, *Science* **140**, 379 (1963).
6. B. Chance, and D. F. Parsons, in preparation.
7. B. Chance and G. R. Williams, *J. Biol. Chem.* **217**, 383 (1955), I.
8. R. W. Estabrook in *Haematin Enzymes* (Symposium, Canberra, 1959), J. E. Falk, R. Lemberg, R. K. Morton, Eds. (Pergamon, Oxford, 1961), p. 436; B. Chance, *ibid.*, p. 597; W. D. Bonner, Jr., *ibid.*, p. 479.
9. D. S. Smith, *J. Cell Biol.* **19**, 115 (1963).
10. B. Chance, *Nature* **169**, 215 (1952); B. Chance and G. R. Williams, *Advan. Enzymol.* **17**, 65 (1956).
11. R. W. Estabrook and A. Holowinsky, *J. Biophys. Biochem. Cytol.* **9**, 19 (1961).
12. A. L. Lehninger, *Sci. Am.* **9**, 62 (1961).
13. D. Pette, M. Klingenberg, T. Bücher, *Biochem. Biophys. Res. Commun.* **7**, 425 (1962); M. Klingenberg and D. Pette, *Biochem. Biophys. Res. Commun.* **7**, 430 (1962).
14. We thank Brigitte Schoener, Reiko Oshino, and Tamiko Yoshioka for technical assistance. Supported in part by grants from the National Science Foundation and the U.S. Public Health Service.

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Diploid and Endoreduplicated Cells: Measurements of DNA

Abstract. *Two-wavelength microspectrophotometry has shown that endoreduplicated cells in an x-irradiated culture of human leukocytes contained about twice as much DNA as diploid cells from the same culture. This supports the assumption that endoreduplicated cells represent a form of polyploidy.*

The doubling of the number of visible chromosome elements, resulting in four-stranded chromosomes, is termed endoreduplication. This phenomenon has been induced in cultures of human leukocytes by various forms of ionizing radiation (*1*). It has been generally assumed that endoreduplication represents a form of polyploidy, that is, that the chromosome set and the DNA content are doubled. It was considered important that this assumption be verified, since the possibility has been entertained that endoreduplication is due to subdivision of multistranded chromatids. Microspectrophotometric measurements were therefore made of the DNA content of normal diploid and of endoreduplicated cells at metaphase in an irradiated culture of human leukocytes.

The leukocyte culture was prepared by modifications of the techniques of Moorhead *et al.* (*2*). Endoreduplication was induced by x-irradiation. Radiation was supplied by a Philips x-ray machine, operated at 15 ma and 250 kv. Filtration was provided by 1-mm Al and 0.25-mm Cu. Dose was measured with a Victoreen condenser roentgen meter and 250-r chamber. The culture was exposed to 400 r of x-irradiation at 202 r/min, 48 hours after it had been prepared. The cells were harvested after another 48 hours, and slides were prepared, which, after drying in air, were stained by the Feulgen procedure, mounted in oil (refractive index 1.545), and sealed with paraffin.

To reduce to a minimum errors due to variations in staining, only one slide was used. This was the slide judged to have the greatest number of suitable metaphase figures. For microspectrophotometric measurements, cells had to be selected which were well separated from other cells. In addition, the chromosomes had to be well bunched together, but not so much that identification of diploidy and endoreduplication would be difficult. This made accurate chromosome counts impossible, but since the cells were apparently intact it was assumed that no loss of DNA material had occurred.

Nine diploid and five endoreduplicated cells fulfilled these criteria. Measurements of DNA were made by the two-wavelength method (*3*) by means of a Canalco microspectrophotometer. The data obtained are presented in Table 1.

From these results it is apparent that the endoreduplicated cells contain more DNA than do diploids, and that their presence, therefore, cannot be due to simple chromatid splitting. Although the confidence limits are fairly wide, the endoreduplicated cells appear to have about twice the DNA content as the diploids. This is consistent with the aforementioned assumption about en-

Table 1. Microspectrophotometric measurements of DNA in diploid and in endoreduplicated cells at metaphase.

Diploid	Endoreduplicated
400	682
430	601
422	797
411	982
431	1081
538	
365	
497	
393	
Mean	
431 ± 18	829 ± 90
95 percent confidence limits	
389–473	597–1079