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THE EFFECTS OF LASER MICROBEAM IRRADIATION ON THE FLAGELLUM OF *CRITHIDIA* (*STRIGOMONAS*) *ONCOPELTI*

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INTRODUCTION

The flagellum of the trypanosomatid flagellate *Crithidia* (*Strigomonas*) *oncopelti* is capable of propagating waves from both its base and tip (Holwill, 1965). In the present study, the capabilities of bend initiation and propagation by the base, tip and points along the flagellum have been studied by observing the effects of irradiation of small regions with a pulsed ruby laser microbeam. The location of the mechanism for deciding the direction of propagation of bends has also been studied by this method.

In most flagella for which the waveforms have been examined, bends are initiated at the base and propagated toward the tip (e.g. Gray, 1955, 1958; Brokaw, 1965); they are not propagated in the reverse direction. In certain flagella of this type, namely the sperm tails of some sea urchins and starfish, the ability to initiate bends appears to be localized in a small region at the base (Goldstein, 1969). Rikmenspoel & van Herpen (1969) suggest that the same may be true for bull spermatozoa, but their evidence is not conclusive.

In some flagella, however, waves can be propagated from either the base or the tip (Walker & Walker, 1962; Afzelius, 1962; Holwill, 1965). This ability suggests that these flagella have, at least, one initiator region at their base and another at their tip, which may or may not be able to act independently of one another. (Alternatively, all points along these flagella may be capable of initiating bends.) In addition, it implies that a mechanism exists for determining which point on a flagellum will initiate bends at any particular time.

The flagellum of *C. oncopelti* has the ability to propagate bending waves in either direction (Holwill, 1965). In contrast to the sperm mentioned earlier, it usually propagates waves along the flagellum from tip to base, and consequently swims with the flagellum pulling the cell body. If the organism is disturbed by, say, swimming into an obstacle, it can reverse the direction of bend propagation. This flagellum has a typical 9 + 2 fibrillar ultrastructure (Holwill, 1965; Burnasheva, Ostrovskaya & Yurzina, 1968).

Different distributions of initiator activity have been suggested by Brokaw (1966*a*) and by Machin (1958, 1963). In the model proposed by Brokaw a single initiator is located at the base, with bending at one point triggering bending at the following point. This model could be modified easily to allow initiation at both the base and tip,

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but it predicts that bends cannot be initiated at other points along the flagellum. In the model proposed by Machin sites arranged along the entire flagellum are capable of bending spontaneously, with the direction of propagation of bends being determined by mechanical constraints at the base and tip. This model predicts that any point on a flagellum may be capable of initiating bends under the appropriate conditions. As mentioned above, in certain flagella which propagate waves only from the base the ability to initiate bends appears, experimentally, to be localized in a small region at the base.

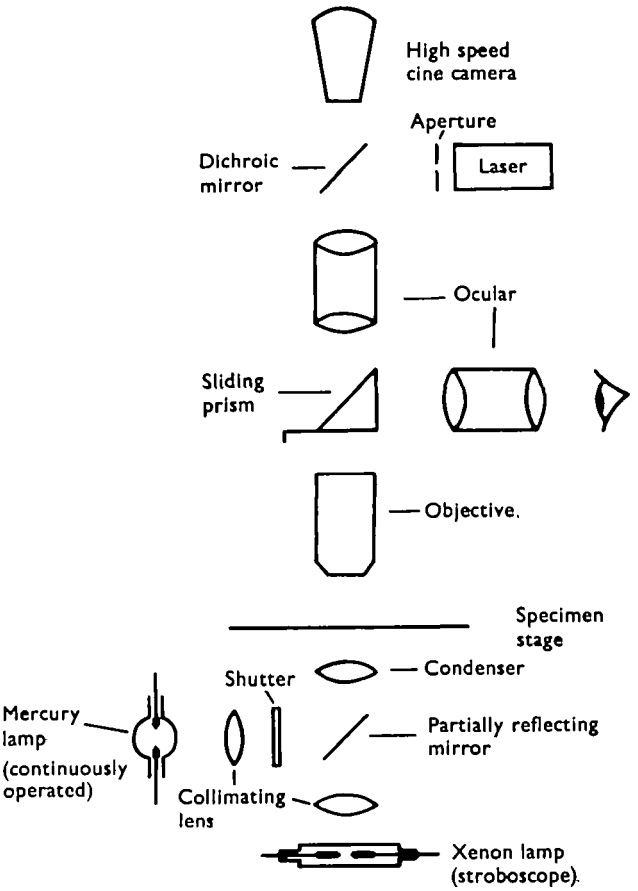
In the present study, small regions of the cell body and flagellum of *C. oncopelti* have been irradiated with a single pulse of a ruby laser microbeam. This has been done in an attempt both to find the sites of initiation and to discover the location of the mechanism which determines the direction of bend propagation.

METHODS AND MATERIALS

Cultures of *C. oncopelti* were grown in 5 cm^3 aliquots in a medium containing 3% proteose peptone, 0·5% glucose and 0·5% sodium chloride (Newton, 1957). The organisms were prepared for use by gently centrifuging them into a pellet, decanting the supernatant medium and re-suspending the cells in distilled water which contained 5 kg m^{-3} of Food, Drug and Cosmetic Blue No. 1 dye and which had been adjusted to pH 7–7·5 with tris (hydroxymethyl) aminomethane. (The dye is necessary for the absorption of laser radiation in the specimen.) In a few experiments this dye solution also contained 0·1–10 mol m^{-3} of adenosine triphosphate (ATP). A drop of the cell suspension was placed on a slide, covered with a coverglass and allowed to stand for perhaps 10 min, during which time a large number of organisms became attached to the glass by their cell bodies with their flagella beating freely.

The apparatus (Text-fig. 1) was similar to that used by Goldstein (1969) except that the results were recorded on cine film (Holwill, 1965) instead of on multiple-exposure frames. A trinocular Zeiss W.L. microscope with a 40× objective was used with either phase-contrast or dark-field optics. Stroboscopic illumination for visual observation was provided by a General Electric FT-230 short-gap xenon flash lamp operated at 1–2 kV and triggered by the amplified trigger output pulses of a General Radio type 1538-A Strobotac (Brokaw, 1963). A Stalex high-speed cine-camera was mounted above the vertical tube of the microscope. The speed control of the camera had been modified to allow photographs to be taken at a constant framing rate. Cinephotographs were taken on 16 mm Ilford FP3 and Kodak Tri-X film at a magnification of 125×, with illumination provided by a 200 W mercury arc lamp (Osram HBO200). Light from this lamp was reflected into the microscope by a partially reflecting mirror placed between the condenser and the xenon flash lamp. A shutter prevented light from the mercury lamp reaching the specimen except during photography. A TRG model 513 biolaser system was mounted directly on the vertical tube of the microscope. The laser emission (wavelength 694·3 nm) was reflected down the tube by a dichroic mirror placed between the camera and the monocular eyepiece, and was focused by the microscope to a nominal diameter of about 2 μm. The laser was aligned by adjusting the mirror so that a hole burned in a thin film of dye coincided with cross hairs in one of the viewing eyepieces (Saks, Zuzolo & Kopac, 1965). A more complete description of the apparatus is given in Goldstein, Holwill & Silvester (1970).

When a suitable organism was selected for study the stroboscopic frequency of the illumination was adjusted to be equal to that of the flagellum so that the flagellum appeared stationary to the observer. The desired point on the image of the flagellum or cell body was then placed under the cross hairs. After this the shutter on the mercury arc lamp was opened to provide illumination for photography and the camera



Text-fig. 1. Schematic diagram of the apparatus.

motor was started. The Strobotac continued to flash in synchrony with the flagellum but was not used to trigger the xenon flash lamp while the camera was running. After a delay of *c.* 1 s, during which the beating of the intact flagellum was filmed, the laser was fired by a trigger output pulse from the Strobotac. The camera was subsequently allowed to run for several seconds to record the effects of irradiation. The films were analysed frame-by-frame on the translucent screen of a PCD digital data reader which had been fitted with a Vanguard analysing projector head. The exact speed of the film during photography was determined from timing marks made on its edge by a neon lamp flashing at twice the 50 Hz mains frequency. The frame immediately after or during which the laser had been fired was usually obvious from the condition of the flagellum, and was further verified by means of a mark made on one side of the film by a neon lamp which was switched on simultaneously with the firing of the laser.

OBSERVATIONS

Flagella usually propagated bends toward the base in the attached cells, although a number of film sequences were obtained in which bends were propagated toward the tip. Flagella of this organism were more resistant to damage by laser irradiation than those of echinoderm spermatozoa (cf. Goldstein, 1969); in addition, a greater degree of damage was desired, either to ensure complete destruction of a region or to sever a flagellum completely. For these reasons a relatively high concentration of absorbing dye was used. The large amount of radiation absorbed often produced a temporary bubble in the irradiated region, which appeared to cause some jarring of the flagellum. The primary effect on the flagellum, however, was probably due to the intense local heating indicated by this vaporization. The irradiated region appeared to be *c.* 2–3 μm in length; the average length of the flagellum is 17 μm (Holwill, 1965). The outline of the flagellum often appeared roughened for several μm beyond the site of irradiation, suggesting possible membrane damage spreading from the irradiated area. Cells often ruptured within several seconds after severe irradiation, even when irradiated near the tip of the flagellum.

Table 1. *Summary of results*

		Behaviour of flagellum	
Wave direction	Laser target	Between body and target	Between target and tip
Tip to base	Tip	Reversal of wave direction	—
	Base	—	Detached, independent propulsion; occasional reversal
	Middle	Reversal of wave direction	Detached, independent propulsion; occasional reversal
Base to tip	Tip	No reversal	—
	Base	—	Detached, independent propulsion; occasional reversal
	Middle	No reversal	Detached, independent propulsion; occasional reversal

The frames reproduced in Plate 1, which illustrates the main effects discussed in the text, have been selected from one of over 100 successful film sequences of irradiated cells. Most sequences were filmed at *c.* 100 frames/s. Table 1 summarizes the results which are discussed in detail below.

Effects on existing waves

In the majority of cases both the velocity and amplitude of existing bends diminished so rapidly that they appeared to decay as standing, or very slowly propagating, waves without reaching the end or irradiated point. However, in about 40% of the cells, bends which were already present in the flagellum continued propagating to the end or to the irradiated point. This was true in flagella irradiated near either the base or the tip, and for propagation in either direction; propagation could continue even in

flagella completely severed near the base. The velocity of propagation of existing bends usually decreased markedly, although in a few mildly irradiated flagella the velocity seemed to remain constant or increase slightly. The precise dimensions of bends were difficult to determine in these flagella, and exact measurements of waveforms were not made.

New bends after irradiation near the tip

Flagella which were propagating bends toward the tip at the time of irradiation continued to do so for at least a few beats. They never propagated bends from the irradiated point. Flagella which were propagating bends toward the base generally immediately stopped initiating beats at the tip. However, a few continued for a few beats even though the tip appeared to be badly damaged. Most flagella then began beating from the base after a pause. This pause was quite variable, lasting from less than 10 ms (one frame period) to several hundred ms, and was related neither to the frequency of the beat nor to the distance from the base to the irradiated point.

Bends propagated toward the tip after irradiation generally had lower frequencies than those of bends propagated toward the base before irradiation. This difference in wave parameters with direction of propagation is also seen in intact flagella (Holwill, 1965).

New bends after irradiation near the base

Those flagella which were propagating bends toward the tip before irradiation usually continued to propagate them in the same direction even when the irradiation was intense enough to sever the flagellum. However, on several occasions the flagellum was seen to initiate bends from the tip after irradiation.

Flagella which were propagating bends toward the base at the time of irradiation usually continued to propagate them in that direction, even when the flagellum was severed. Occasionally a flagellum propagated bends toward the irradiated point for a few beats, paused and propagated a few beats toward the tip. The length of the pause was variable. This occurred even in fragments of severed flagella. An example of this is shown in Plate 1.

Fragments of flagella severed from the cell could continue beating for up to ten complete cycles. This number was not increased by the addition of ATP to the suspension medium.

Effects of irradiation near the centre of the flagellum

Organisms irradiated at a point about halfway along the flagellum exhibited essentially the same effects as those irradiated near the ends. The region proximal to the irradiated point propagated bends from the base after a pause. The region distal to the irradiated point could propagate bends in either direction and was capable of the same reversal of direction of bend propagation as in flagella irradiated nearer the base.

Effects of irradiation of the cell body

Flagella continued beating for a number of cycles after an irradiation which was severe enough to burst the cell body. Often the frequency of the beat increased very rapidly after irradiation, and when bend propagation was formerly from tip to

base it sometimes reversed after several cycles. The delay between irradiation and reversal of bend propagation was generally greater following irradiation of the cell body than following irradiation of the flagellum.

DISCUSSION

When either a region near the base or one near the tip of the flagellum was irradiated, the other end was capable of assuming the function of initiation of bends. (Before discussing this further we should point out that the flagellum of this organism emerges from a deep invagination in the cell body (unpublished results) so that irradiation near the base was always at least a few μm away from the basal structures.) This ability indicates that each of these regions can act independently of the other. The ability of isolated pieces of flagellum to initiate bends at the severed region indicates that regions other than the base and tip can act as initiators. Although the ability to initiate bends was not confined to the ends, these results do not distinguish between the possibilities that (i) bends can be formed anywhere along a flagellum and (ii) bends can be formed at a small number of specialized regions. However, since an intact flagellum propagates bends from its ends the arrangement of a small number of apparently unused specialized regions seems unlikely. It seems more reasonable to interpret these experiments as indicating that, in order to allow bend propagation in either direction, this flagellum is modified so that essentially any region along it is potentially able to initiate bends, although one end of the flagellum is generally dominant.

Our experiments do not indicate the minimum length of a region necessary to produce bends. However, observations on other flagella (Brokaw, 1965 and unpublished observations) indicate that no more than a few μm are necessary.

Although the ultrastructure of this flagellum has not yet been extensively studied, it is known to have a typical 9 + 2 fibrillar ultrastructure (Holwill, 1965; Burnasheva *et al.* 1968). The ability of intermediate points to initiate bends along this flagellum therefore indicates that the complex structures generally observed at the base of flagella are not necessary for bend initiation and suggests that the evident specialization in the basal region has other purposes. However, one should note that regions proximal to an irradiated point *always* beat from the base, thus suggesting that this region has a dominant role.

Other organisms that have been studied by the present technique, such as echinoderm spermatozoa (Goldstein, 1969), appear incapable of initiating bends from points other than the base. Flagella of these organisms have a typical 9 + 2 fibrillar structure (Afzelius, 1959) and, although the ultrastructure of the flagellum of *C. oncopelti* has not yet been extensively studied, it is known to have a similar appearance in the electron microscope (Holwill, 1965; Burnasheva *et al.* 1968). This similarity suggests that there is little ultrastructural difference between flagella in which the intermediate regions are capable of initiating bends and those in which these regions can only propagate them.

When reversal of propagation occurred, the delay between irradiation and reversal was quite variable. This variability may have been due to variations in the degree and quality of the damage caused by irradiation. The minimum delay observed was

c. 10 ms or less, which is significantly less than the propagation time of a bend along a flagellum. This observation, and the effects of irradiation on bends which were already present at the time of irradiation, indicate that some form of signal exists which travels substantially more rapidly than the bends. Several types of signal are possible. For example, the signal might be due to a shock wave, or to membrane damage spreading from the irradiated point, or to an electrochemical stimulus similar to a nerve impulse. An alternative possibility, which is supported by observations on echinoderm spermatozoa, is that the signal is produced by the interruption of some internal mechanical activity associated with bending, such as, perhaps, the sliding of the outer filaments (cf. Satir, 1968). This could clearly affect a remote region along the flagellum more rapidly than the passage of a wave to that region.

The response to localized irradiation of flagella which propagate waves only from the base is closer to that predicted by a model of localized bend propagation (Brokaw, 1966*a*) than to that predicted by a model of coupled oscillators arranged along a flagellum (Machin, 1958, 1963). On the other hand, the response of the flagellum of *C. oncopelti* is closer to that predicted by a model of coupled oscillators, any of which is able to initiate bends. However, its response differs from the prediction of Machin's model that propagated bends should begin as standing waves which develop into travelling waves as the amplitudes increase and the non-linearities in the system become important. In the flagellum of this organism bends appear to begin *de novo* from an end or irradiated point. Bends also appear to start at the ends of the intact flagellum without development of a standing wave (Holwill, 1966).

The occasional reversal of the direction of propagation of bends in isolated flagellar segments indicates that the influence of the cell body is not necessary for reversal. The relatively long delay between irradiation of the cell body and reversal of the propagation direction lends weight to this inference. These results do not, of course, exclude the possibility that the cell body is involved in some form of control of flagellar bending.

Flagella of *C. oncopelti* beat for up to ten cycles after being severed, implying that they store enough energy for at least ten beats. Brokaw (1965 and unpublished observations) has observed sperm tails beating vigorously for several seconds after being separated from the mid-piece; indicating that these flagella also contain enough energy for many beats. Passive diffusion of ATP from mitochondria in the cell body near the base has been suggested as a possible means of supply of energy (Brokaw, 1966*b*; Raff & Blum, 1968; Nevo & Rikmenspoel, 1970).

Nevo & Rikmenspoel (1970) have calculated the minimum amount of ATP distributed along a flagellum by a simple diffusion gradient, assuming that the concentration falls to zero at the tip. This estimate is probably slightly too small; kinetic considerations indicate that the concentration should not fall to less than about 0·3 mol m^{-3} for a flagellum beating 30 times a second (Silvester & Holwill, 1965). Using the figures of Nevo & Rikmenspoel for sea-urchin spermatozoa, a flagellum contains at least $1{\cdot}0 \times 10^{-18}$ mol of ATP. The rate at which ATP is used by a sperm flagellum is *c.* $1{\cdot}0 \times 10^{-19}$ mol s^{-1} (Brokaw & Benedict, 1968). Assuming that these figures are generally applicable a diffusion mechanism would enable an amputated flagellum to store enough ATP for about 10 beats.

This figure is, perhaps fortuitously, close to the 10 beats exhibited by some flagellar segments after amputation. This observation alone might suggest that the cessation of beating after irradiation is due simply to chemical depletion of the flagellar ATP by dephosphorylation. However, more efficient use of ATP by means of an adenylate kinase could provide a larger reservoir of energy (Raff & Blum, 1968), and if this were the case chemical depletion alone would not account for the limited number of beats. Although in the normal dye solution irradiation damage may have allowed the physical depletion of ATP by diffusion out of the flagellum, it is unlikely that this would have occurred in the ATP-rich dye solution, where beating also stopped a few cycles after irradiation. The latter observation indicates that the cessation of beating may be due to some other cause than the absence of ATP—for instance, it may be due to a depletion of some intermediate, either in the dephosphorylation cycle or in an active transport system, if such exists.

From this study of *C. oncopelti* we have been able to infer that, for flagellar activity in this organism, neither a unique region of the flagellum nor the cell body is necessary, and that regions able to initiate waves in this flagellum are probably not dissimilar in structure from regions which only propagate waves in flagella of other organisms. The present results allow no firm conclusions to be drawn about the mechanism of energy supply to the flagellum, but do suggest that flagellar activity involves the transmission of a signal along the flagellum at a greater velocity than that of wave propagation. The mechanical behaviour of this flagellum is closer to that predicted by the Machin model than by any other model so far proposed for bend propagation.

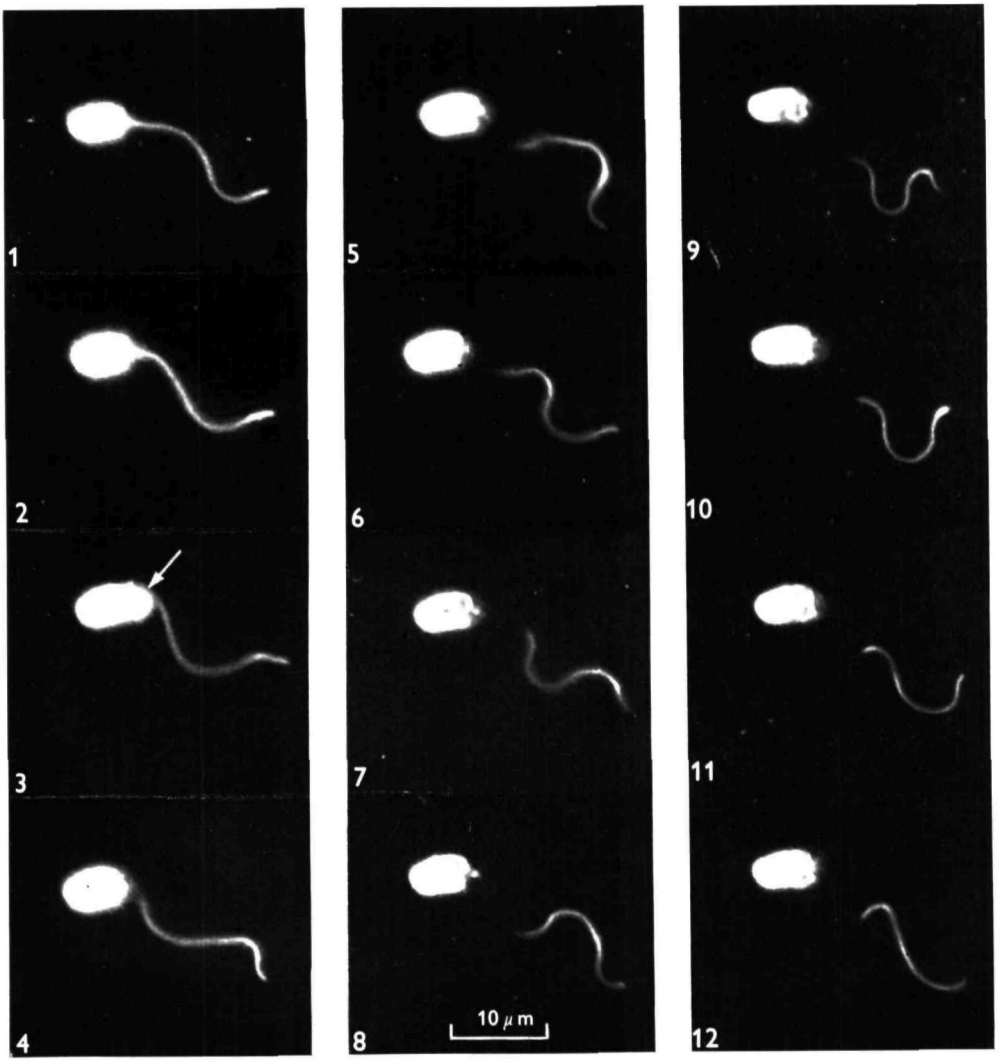
Clear differences exist between the behaviour of the flagellum of *C. oncopelti* and the flagella of certain echinoderm spermatozoa, which have also been examined by a similar method (Goldstein, 1969). For this reason it is evident that one should exercise caution in giving the above conclusions a wider validity than they merit at this stage. It will be interesting to see if these differences arise because of the different directional characteristics of waves on the two types of flagella or whether flagella that propagate waves unidirectionally can exhibit some of the properties described in this paper.

SUMMARY

1. Flagella and cell bodies of *Crithidia* (*Strigomonas*) *oncopelti* were irradiated at preselected points with a pulsed ruby laser microbeam. Results were recorded by high-speed cinephotomicrography. A flagellum could be completely amputated at the irradiated point.

2. The portion of the flagellum between the cell body and the irradiated point beat from the base after irradiation. The amputated portion of the flagellum could beat from either the tip or the irradiated point, and could beat first from the tip and then from the irradiated point or vice versa. Beating could continue for up to ten cycles.

3. For flagellar activity in this organism neither a unique region of the flagellum nor the cell body is necessary. Wave propagation appears to involve the transmission of a signal at a greater velocity than that of the wave. The results favour a model of bend propagation which allows for a distribution of autonomous initiators along the flagellar length.



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EXPLANATION OF PLATE

Selected frames from a cine-film illustrating the effect of laser radiation on a flagellum of *Crithidia oncopelti*.

Figs. 1, 2. Waves propagating from tip to base before irradiation.

Figs. 3, 4. The laser beam hits a region near the base and severs the flagellum from the cell body. The disturbance produced by the laser beam is arrowed.

Figs. 5–8. Following irradiation, bends continue to propagate from the tip of the flagellum.

Figs. 9–12. At a later time the direction of wave propagation reverses.

(Elapsed time (ms): Fig. 1, 0; 2, 10; 3, 20; 4, 30; 5, 120; 6, 160; 7, 200; 8, 230; 9, 900; 10, 930; 11, 970; 12, 1020.)