Nagy *et al.* As noted, their morphology is simple, and they appear to lack all the other properties suggestive of a biological origin that were previously attributed to them: fluorescence in ultraviolet, biological staining, and insolubility in acids. The density of most of these particles is high, indicating a predominantly inorganic composition. The case for a biological origin thus rests entirely on their featureless morphology. It would seem that further, independent criteria must be developed before a biological origin of these particles can be safely postulated.

Particles of the second class have a complex morphology; some fluoresce in ultraviolet; many take biological stains; others reveal their organic composition by their low density or their insolubility in acids. All these properties speak strongly for a biological origin. However, these particles are exceedingly rare. They have not been found in our samples of the Orgueil meteorite. We cannot completely rule out the possibility that this discrepancy is due to sampling error, although some of our material came from the very stone studied by Claus and Nagy. None of these particles have been conclusively identified in thin sections, and the particles in thin sections most nearly resembling them appear to be artifacts. Many of them show a morphological resemblance to common airborne contaminants. None of the particles of this class seem to have been observed by two or more independent workers; neither Staplin (*10*) nor Palik (*8*) mention particles of the type described by Nagy *et al.*, although they do report other highly structured particles. Thus, the particles of the second class pose a problem exactly opposite to that posed by those of the first class: although it is almost certain that they are of biological origin, it is very unlikely, in the majority of cases, that they are indigenous to the meteorite. It is unfortunate that Nagy and his associates fail to make a distinction between these two classes and thus give the impression that the striking properties of a few particles of the second class (for which contamination was not ruled out) also pertain to the much more numerous, probably indigenous, particles of the first class.

We believe that the problem of organized elements merits further investigation. However, our work shows that the problem is a much more difficult one than has been implied in previous publications. The particles of the first class are in a morphological no-man's-land, and to establish their possible biological origin, new techniques and new criteria will have to be developed. As for particles of the second class, proof must be given that they are not terrestrial contaminants. This, like all negative proof, may be very difficult to obtain, unless present techniques are improved very greatly (*26*).

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27. The fly ash sample was obtained through the courtesy of Drs. P. H. Gregory and H. E. Crossley.

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# Electron Microscope Studies of Ribosomal Clusters Synthesizing Hemoglobin


Jonathan R. Warner, Alexander Rich, Cecil E. Hall


Ribosomes are RNA-protein particles which have a diameter of approximately 230 A in the electron microscope. For many years it has been believed that they are the site of protein synthesis. However, in studies with rabbit reticulocytes, we have been able to show that the site of hemoglobin synthesis in vivo is not the single ribosome but rather a cluster of ribosomal particles, which we have called a "polyribosome" or simply a polysome (*1*). Somewhat similar observations have also been made by Gierer (*2*). In this paper we describe electron-microscopic studies of this protein-synthesizing structure.

The isolation of this larger unit in protein synthesis rests heavily on recognizing that it is extremely fragile and is easily broken up by mechanical agitation or by enzymatic activity. By using reticulocytes, one can gently lyse the cells in hypotonic buffer and thereby obtain a lysate which has in it all of the components of the hemoglobin-synthesizing cell without having subjected the cell contents to degradation from shear because of too much manipulation. The existence of the polysome was first demonstrated by incubating the cells in vivo with a mixture of $C^{14}$-labeled amino acids and then lysing the cells. One ml of this lysate was layered on top of a 15 to 30 percent sucrose density gradient in a SW 25 Spinco ultracentrifuge tube and then spun for approximately 2 hours. After this, the tube was punctured and fractions were collected and analyzed. The fractions obtained in this way were kept for electron-microscopic studies. As shown in Fig. 1, the optical density shows two peaks, one of which occurs with a sedimentation constant of 76 S and is associated with the single ribosomal units.


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The other peak is lower and much broader, and accounts for approximately one-third of the total ribosomal optical density; the material from its peak sediments at 170 S. However, as can be seen in Fig. 1, virtually no radioactivity is associated with the single ribosomal peak sedimenting at 76 S, and the bulk of the radioactivity is associated with the more rapidly sedimenting peak at 170 S. Thus, the rapidly sedimenting material is the site of protein synthesis.

In our previous paper we showed that it is possible to degrade the faster peak into a series of discretely sedimenting units (*1*). The 76 S unit is a single ribosome. The diribosomal unit sediments at 108 S, the triribosomal unit at 134 S, the tetra- at 154 S, and finally the penta- at 170 S. However, the breadth of the 170 S peak suggests that it consists of a mixture of polysomal units, the predominant species being the pentamer. Its position on the gradient is designated by an arrow in Fig. 1.

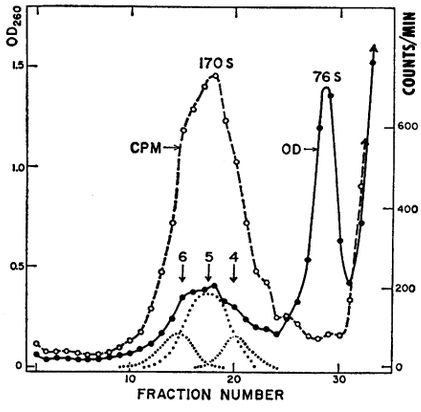


Fig. 1. One ml of packed rabbit cells containing over 90 percent reticulocytes was suspended in saline solution (0.14*M* NaCl, 0.005*M* KCl, 0.0015*M* $MgCl_2$) so that the final volume was 1.5 ml, after the addition of 1 mg $NaHCO_3$, 2 mg glucose, and 0.08 mg $Fe(NH_4)_2SO_4$, and incubated at 37°C. After 15 minutes incubation, 4 $\mu$c of $C^{14}$-labelled amino acids from an algal hydrolysate which had an activity of 1.6 $\mu$c/mg were added; 45 seconds later the mixture was chilled by adding 25 ml of cold saline, and the cells were pelleted by low speed centrifugation. Osmotic lysis was carried out by adding 9 ml of standard buffer solution to the cells, and the cell walls were removed by centrifugation at 10,000*g* for 15 minutes. One ml of lysate was used for the sucrose gradient as described in the text. Thirty-six fractions were collected. The protein was precipitated by adding carrier serum albumin and trichloracetic acid. It was collected on Millipore filters, and counted in an end-window geiger counter. The arrows labeled 4, 5, and 6 represent the sedimenting positions of ribosomal tetramers, pentamers, and hexamers. The dotted lines schematically represent their distribution in the gradient. Counts per minute are shown by the dashed line and $OD_{260}$ by the solid line.

Samples were prepared from such sucrose gradients for electron microscopy. As pointed out earlier, the 78 S peak showed a typical field of single ribosomes (*1*). A sample of the 170 S peak was prepared for electron microscopy by placing a droplet of the solution on a silicon-monoxide coated electron-microscope grid for a short time period at 4°C. The droplet was taken directly from the sucrose gradient and, accordingly, it was necessary to wash off the droplet in order to free the surface of sucrose. This was done by gently rinsing the electron-microscope grid with successively decreasing concentrations of sucrose in standard buffer solution (0.01*M* KCl, 0.0015*M* $MgCl_2$, 0.01*M* tris buffer, *p*H 7.4). Rinsing directly with a buffer solution tended to disrupt the ribosomes but a gradual decrease in sucrose concentration preserved their structure. This effect may be associated with the very large hydration of ribosomes (*3*). At the end of this process the electron-microscope grid was rinsed with the buffer solution alone and then dried in air. It was shadowed with platinum at a shadowing angle of 5:1 and then photographed in an RCA EMU 3 Electron Microscope at a magnification of 16,000. A typical field is shown in Fig. 2.

Most of the ribosomes on the field appear as clumps, usually in groups of five. Occasional single ribosomes appear. However, since the sample was obtained from the 170 S peak, the single ribosomes must have been produced through the breakdown of a larger unit in the course of sample preparation. Electron micrographs were prepared from samples obtained throughout the band region. These fields show that while pentamers are more common in the center of the peak, tetramers become increasingly common in the part of the peak that sediments more slowly, and hexamers are more common in the leading edge of the peak. Their distribution is shown qualitatively in Fig. 1 by the three dotted curves lying under the optical density peak. The arrows labeled 4, 5, and 6 correspond to the approximate sedimentation positions for tetramers, pentamers, and hexamers.

An attempt was made to obtain more quantitative information about the actual distribution of polysomes in the peak. For this purpose nine different fields were counted, including approxi-

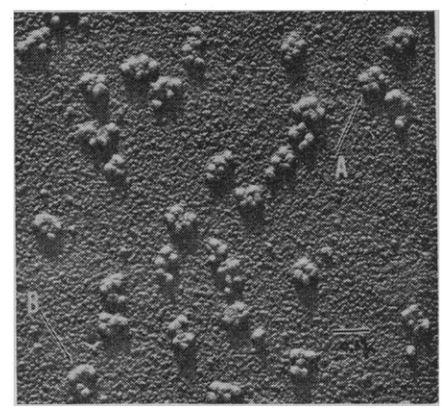


Fig. 2. Electron micrograph of the material sedimenting at 170 S, under arrow 5 in Fig. 1. Most of the ribosomes appear in clusters which are compact (*A*) or are more open (*B*). The horizontal mark indicates 1000 Å.

mately 5500 ribosomes from the peak tube (under arrow 5 in Fig. 1). The fields were scored in two ways. One described the number of ribosomes in each group and the other described the configuration of the group. Approximately 75 percent of the ribosomes are found in pentamers while about 10 percent each are found as tetramers or hexamers; the remainder is scattered among rare septamers and mono-, di-, and trimers (Fig. 3). It is reasonable to believe that the monomer, dimer, and trimer arise from breakdown products resulting from sample preparation. However, it is quite likely that the appreciable number of tetramers and hexamers represents the actual distribution of these units in the center of the 170 S peak rather than artifacts of the preparation. The preparation has been treated so gently that it is quite

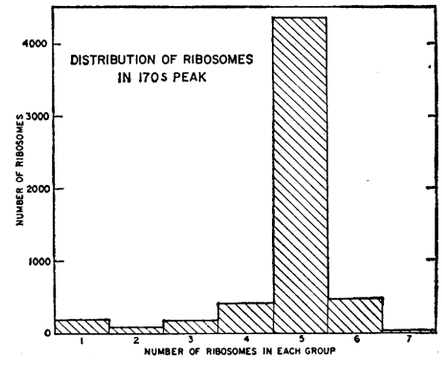


Fig. 3. The distribution of ribosomes in groups was measured in nine large fields similar to Fig. 2. The number of ribosomes in each group was counted over a total of 1300 groups including 5500 ribosomes. Over 75 percent of the ribosomes were in pentamers.

unlikely that these could have arisen by chance. Furthermore, an understanding of the mechanism whereby polysomes function in protein synthesis must explain this natural distribution in vivo.

Treatment of the polysome with very low levels of ribonuclease at 4°C rapidly broke down the larger structure into individual ribosomes which sediment at 76 S (*1*). Only under those circumstances was radioactivity associated with the monomer peak. Other procedures such as grinding with alumina or centrifugation to form pellets and resuspending resulted in a similar breakdown of the polysome structure. Thus, this is a very fragile structure in which the ribosomal units are held together by RNA.

Considerable interest is associated with the configuration of the polysome. Many of the clusters which are seen in Fig. 2 are tightly clumped. However, the ribosomes are not in direct contact since the distance from center-to-center between adjacent ribosomes is usually 300 to 350 Å, while the diameter of the individual ribosome is approximately 230 Å. This suggests that the sphere-like ribosomal units may be held together by a very thin filament of RNA, and therefore, it is quite possible that the polysome may assume a variety of configurations. Indeed, this is indicated by our statistical study. In the survey two different types of configuration were noted. One of these is tightly clumped, with all five of the ribosomal units in close contact with each other, as shown by *A* in Fig. 2. The other configuration is more extended as shown by arrow *B* in Fig. 2. In the fields that we studied, approximately 25 percent of the polysomes were in an open configuration and 75 percent were in a tightly clumped configuration. During the preparation of the sample the ribosomes are deposited by gravitational action or convection after the droplet is placed on the electron-microscope grid. Once the ribosomes descend and make initial contact with the supporting film, they are undoubtedly pulled down by surface forces. If the polysomes existed in a more or less extended chain configuration and all of them struck the silicon-monoxide surface at approximately the same time, then they might maintain their extended configuration. However, it is more probable that one of the ribosomes at the end or the middle of the group touched the silicon monoxide surface first and the other ribosomes were then pulled down quite close to it. In short, it is not unreasonable to believe that some of the ribsomal clumping may be a result of the method of preparing the specimen for electron-microscope observation rather than an intrinsic property of the polyribosomal unit.

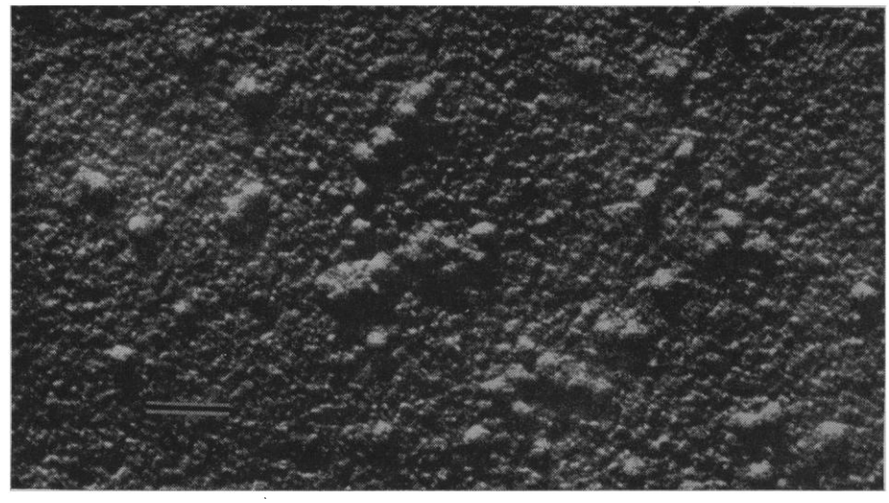

Fig. 4. Electron micrograph of an unfractionated lysate of rabbit reticulocytes. The granularity of the background is due to a carpet of hemoglobin 60 to 70 Å thick. The ribosomal clusters can still be seen and are more extended than in Fig. 2. The horizontal mark is 1000 Å.

In order to obtain more information about the configuration of the polysomes in vivo, electron-microscope studies were carried out directly on the lysate without any purification of polysomes. Thus, a droplet of lysate was placed directly on the electron-microscope grid, allowed to remain there briefly at 4°C, and then washed off with the standard buffer. During this short interval a large amount of hemoglobin was deposited on the grid, together with individual ribosomes and the ribosomal clusters. An example of an electron micrograph of a lysate is shown in Fig. 4. The background is much rougher than in Fig. 2, since it consists of a carpet of hemoglobin. The thickness of this layer can be calculated directly by measuring the length of the shadow cast by ribosomes in this figure in contrast to the much longer shadows which are observed in Fig. 2. From this we conclude that the hemoglobin carpet is about 60 to 70 Å thick, which suggests that the layer is approximately one molecule thick. Despite the roughness of the background, the polyribosomes can still be seen. However, in the lysate a much larger percentage of these clusters are in a linear, almost extended form as shown in Fig. 4. Here the individual ribosomes in the ribosomal cluster are less likely to be adsorbed on to each other as they settle on the silicon-monoxide surface because of the large concentration of hemoglobin molecules that are deposited at the same time and may serve to prevent the condensation of the rather flexible string of ribosomes. Thus, our conclusions from the electron-microscope studies of the entire lysate are twofold. The polysome structure can be seen directly without isolation; its existence is thereby confirmed. Secondly, since a larger number of these clusters appears in a linear array the natural configuration in vivo might be an extended array rather than a tightly clumped group held together in a circular configuration. However, we are making electron-microscope studies of thin sections of reticulocytes to learn more about the configuration within the cell.

Electron micrographs such as shown in Fig. 2 occasionally show the polysome oriented in a fashion favorable for disclosing the existence of a thin bridge connecting the ribosomal units. This thread-like structure, however, cannot be seen consistently in that type of preparation because of the heavy shadowing of platinum and the roughness of the background which arises from the persistence of sucrose from the original droplet. However, we have obtained micrographs with a very clear background by shadowing samples on a freshly cleaved mica substrate. For this technique (*4*) the polysome preparation was dialyzed extensively against a standard buffer solution to eliminate the sucrose. The material was then sprayed in micro-droplets onto a freshly cleaved mica surface which was

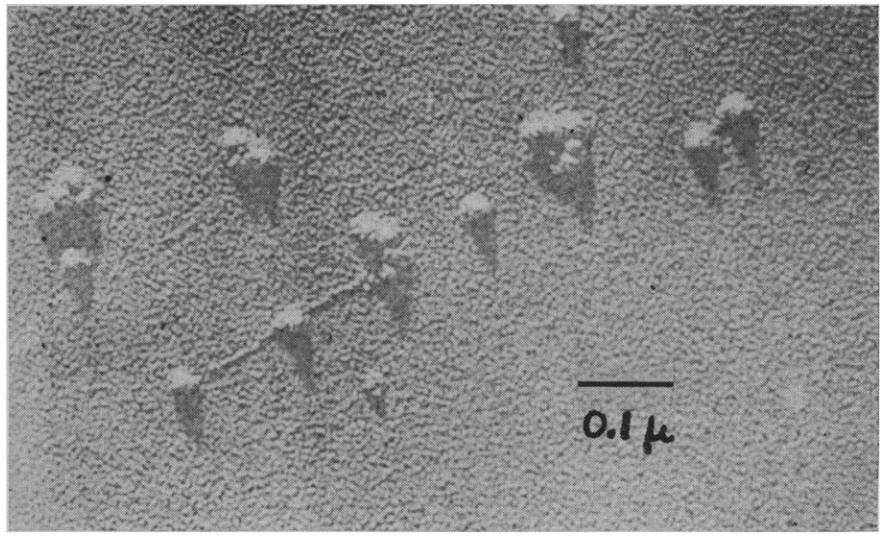


Fig. 5. A sample from the 170 S peak was dialyzed and sprayed on mica as described in the text. The electron micrograph has a smooth background and two 10 to 15 Å strands can be seen connecting ribosomal units of disintegrated polysomes.

then shadowed with platinum and backed with a supporting film that was subsequently stripped and mounted. A sample of this preparation is shown in Fig. 5. The dialysis followed by spraying causes considerable degradation of the polysomal structure. However, these disintegrating polysomes frequently show thin threads, 10 to 15 Å diameter, as illustrated in Fig. 5. Here two single ribosomes can be seen attached to two thin threads each approximately 1300 Å in length, one of which ends in a group of three ribosomes. These threads could not arise through the disintegration of individual ribosomes, since control spray preparations of single ribosomes do not show these threads. These are breakdown products, since the sample itself was prepared from material which sediments rapidly at 170 S. Accordingly, we believe that these threads may represent the RNA strand which holds the ribosomes together to form the polysome. The diameter is consistent with their being a single strand of ribonucleic acid.

Positive stain and negative stain preparations were made with uranyl acetate and phosphotungstic acid, respectively, for examination with the electron microscope. A sample of a negative stained preparation is shown in Fig. 6. The individual ribosomal units in the polysome are more clearly outlined than in the shadowed preparations. In addition, the ribosomes are separated from each other by a distance of 50 to 100 Å. Uranyl acetate stains nucleic acid specifically and the micrographs of such preparations show a series of discrete, dark round objects in arrays, usually with no connecting bridge. However, in some cases very thin uranyl-acetate-staining bridges can be seen connecting the darker ribosomal units. These positively staining bridges are consistent with the existence of a thin filament of ribonucleic acid which holds the ribosomes together.

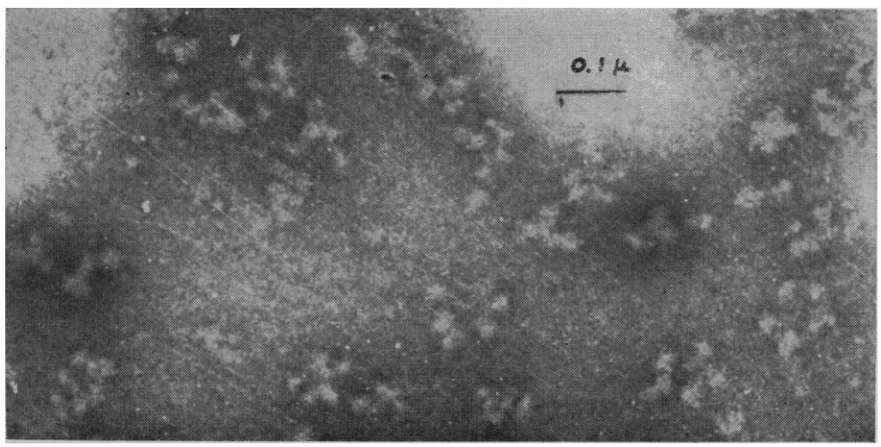


Fig. 6. A sample from the 170 S peak was prepared by negative-staining methods (*6*). The polyribosomes can be seen with the ribosomal subunits separated by gaps of 50 to 100 Å. The horizontal mark indicates 1000 Å.

Measurements have been made of the separation from center to center between the nearest neighbor ribosomal units on the chain. In shadowed preparations, this distance is 280 to 350 Å, including both the lysate and purified preparations. In the negatively stained preparations separations are about 340 Å, while in the positively stained preparations the separations are often somewhat further apart.

*Interpretation.* We have demonstrated that the polysome is the site of hemoglobin synthesis and that the predominant species contains five ribosomal units (*1*). Here we have explored quantitatively the distribution of ribosomal units in the polysome peak and have presented evidence for an intrinsic heterogeneity in the number of ribosomes which are found in the polysomal units. Mainly pentamers are found at the peak which sediments at 170 S, along with a smaller number of tetramers and hexamers. In our earlier work we demonstrated that the ribosomal units are held together by RNA. The strands which are seen in the polysomal preparation are consistent with the existence of a single strand of RNA that holds the ribosomes together. As discussed previously, we think it is quite likely that this strand is, in fact, the messenger RNA strand which contains the information for assembling the amino acids in hemoglobin (*1*). In experiments on the interaction of a synthetic messenger, polyuridylic acid with bacterial ribosomes, several investigators (*5*) have described the formation of rapidly sedimenting peaks, which we take to be polysome formation.

Hemoglobin consists of four polypeptide subunits, each of which contains approximately 150 amino acids. If we assume a coding ratio of 3 and a translation of 3.4 Å per nucleotide, we would anticipate a messenger-RNA strand of approximately 1500 Å. This is approximately the contour length which we observe in the polysome containing 5 ribosomes with a diameter of approximately 230 Å and a gap of 50 to 100 Å between the ribosomal sub-

units. If the ribosomes are held together by the hemoglobin messenger-RNA strand, then it is possible that the ribosomes attach to the messenger at one end, gradually make their way along the messenger RNA as the polypeptide chain grows longer and finally detach at the opposite end. This attachment and detachment of ribosomal units may not be synchronous (*1*) and, therefore, it would produce a natural distribution in vivo which contained predominantly pentamers and also a smaller number of tetramers and hexamers. Thus, such a mechanism may account for the actual distribution of polysomes which is observed in the preparation. Furthermore, the spacing between the ribosomal units appears to be the same in the fresh lysate as in the negatively stained preparations. This distance seems to be fairly constant with an interribosomal gap of approximately 100 Å. However, in some cases the gap may be as much as 150 Å. Futhermore, the separation between adjacent ribosomes in an individual array is not always uniform. This divergence in the separation between ribosomes suggests that the postulated movement along the messenger strand is not completely synchronous but may be statistical in nature (*7*).


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3 December 1962


## Skin Resistance Recording in the Unrestrained Rat


*Abstract.* The level of basal skin resistance is proposed as a measure of motivation. It is relatively simple to obtain and can be measured concurrently with behavior of interest to the experimenter. The technique described requires a grid floor, but the assessment procedure does not affect the organism's state because of the subthreshold measuring current that is used. This method in no way restrains the rat and can therefore be used in an unlimited number of experimental situations.


Skin resistance in the form of specific responses (galvanic skin response), nonspecific fluctuations, and basal resistance level (BRL) has been used as an index of aspects of human motivation for many years. Levy and his coworkers (*1*) have added to these a continuous BRL measure obtained by using a very slow write-out and relatively less-sensitive recording, thus emphasizing the slow, less-transient shifts in skin resistance, as contrasted to traditional techniques of measuring galvanic skin response (GSR).

Lykken and Rose (*2*) have described a device for measuring GSR in rats, but have not presented any data obtained with the apparatus. Their method seriously restrains the rat: the tail must be taped down and each foot is inserted through a slot which connects it to the electrode jelly. Because of the postural restrictions and the stress caused by severely restraining the rat, the number of experimental situations in which their "rat-holder" can be used is limited. It is likely that their assessment procedure affects the skin-resistance values that are obtained.

Our report describes a technique for measuring skin resistance (BRL and GSR) in the unrestrained rat. The animal is placed on a grid floor, and a subthreshold (10 $\mu$amp) current is passed through this grid. Fluctuations in the rat's resistance appear as voltage changes which are amplified and recorded.

A servorecorder with suitable modifications is used to provide the constant measuring current, to amplify, and to record the animal's resistance. The instrument has a rectilinear write-out on a wide band (5 inches). This permits accurate and easy reading at both extremes of the scale as well as simultaneous recording of the absolute resistance (BRL) and momentary changes (GSR). Depending on the chart speed, the GSR component can be seen more or less readily. At a speed of 4 in./min, for example, resistance changes in response to a specific stimulus can be seen very clearly. At a speed of 6 in./hr, however, a record is obtained which indicates the general alertness pattern, somewhat comparable to the tracings presented by Levy *et al.* Since zero resistance is indicated at the bottom of the record, all resistance levels may be read directly and comparisons between animals are readily obtainable.

Readings obtained with hooded rats (90 to 365 days old) range between 50 kohm and 2 Mohm, depending on the situation. Because the rat makes and breaks contact with the grid as it moves, the resistance level of the rat may momentarily approach near infinite resistance. This has required the addition of a current limiter into the circuit which establishes a scale that is linear over three-fourths of its span and compresses the remaining possible readings into the top fourth of the scale. By using a suitable calibration procedure, resistance values can also be obtained over the nonlinear portion of the scale.

It might seem that the noise produced by the animal's movement would completely overwhelm the signal (possibly this assumption accounts for the

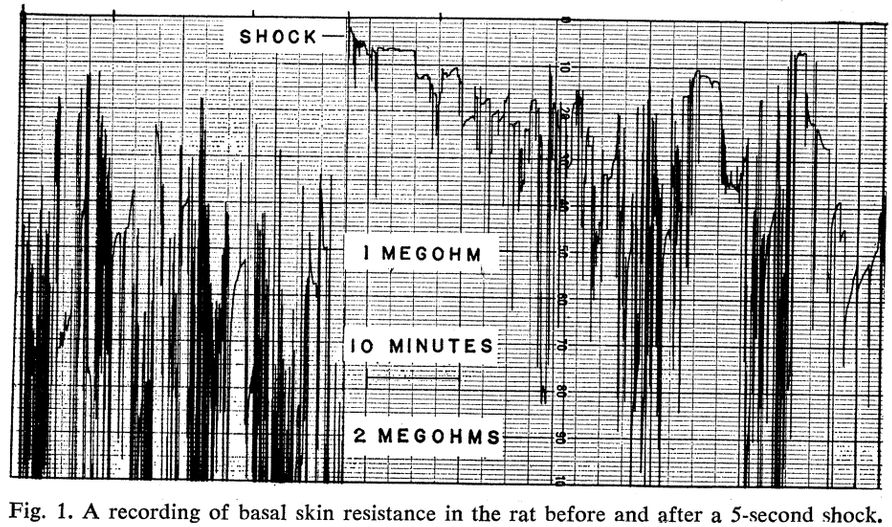


Fig. 1. A recording of basal skin resistance in the rat before and after a 5-second shock.