Molecular Entomology Symposium, July 109-124, 1990

THE USE OF ROCKET IMMUNOELECTROPHORESIS IN THE STUDY OF INSECT HAEMOLYMPH PROTEINS

M. L. PAN[1] and W. H. TELFER[2]

1. *Department of Zoology, University of Tennessee, Knoxville, TN 37996-0810;*
2. *Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018*

INTRODUCTION

In the past two decades, rocket immunoelectrophoresis (RI) (Laurell, 1966) has been a method of choice for the detection and quantitative determination of proteins (Gross and März, 1988), and many insect biologists have adopted it as well. RI can easily quantify 50 ng of protein; this level of sensitivity of detection, not even considering its specificity, makes it superior to those of general protein determination techniques (Davis, 1988). Recently, we compared Oudin's immunodiffusion technique (Telfer and Williams, 1953) and RI in the quantification of five haemolymph proteins endocytosed by the oocytes of the cecropia silkmoth, and found their agreement to be excellent (Telfer and Pan, 1988). RI has the distinct advantage that only a small sample volume, e.g., a few microliters, is required for analysis, while the Oudin technique requires much larger sample volumes. Thus, with a small-bodied insect, or when sample volume is limited, the choice is obvious. We demonstrate here the versatility of RI by discussing how we have used it to 1) characterize a complex antiserum, 2) measure the concentrations of two very different antigens, and 3) study the immunochemical relationships of vitellogenins of three saturniid moths. For other applications of RI, see Axelsen *et al.* (1973).

GENERAL PRINCIPLES OF ROCKET IMMUNOELECTROPHORESIS

RI is an electrophoretic modification of Mancini's method for single immunodiffusion on glass plates, in which antigen diffuses radially from sample wells bored into a layer of agarose containing antibody (Mancini *et al.*, 1965). RI, rather than relying on diffusion, drives a disc-shaped spot of antigen electrophoretically from the sample well into the antiserum-agar. The buffer used in RI has a pH (8.6) at which the low mobility of the antibody is balanced by electroendoosmosis, so that during electrophoresis the antibody remains stationary in the gel. To yield rockets, the isoelectric point of the antigen must be significantly different from that of the antibody (most proteins in fact have more acidic pI's) and the

concentration of the antigen in the sample well must be in large excess of the equivalence concentration of the antibody in the surrounding gel. Under these conditions, the antigen electrophoreses through the gel as a focussed, ellipsoid zone which, at its center, saturates the antibody, forming soluble complexes. On the right and left edges of its electrophoretic pathway, by contrast, the antigen is below equivalence concentration. (The distribution of the antigen across the zone is actually bell-shaped.) The travelling ellipse thus leaves behind a thin line of precipitate along each side of its electrophoretic path. Precipitation also occurs at the migrating front of the ellipse, which can be visualized as an arc connecting the growing ends of the side lines, if electrophoresis is prematurely interrupted. As free antigen and soluble complexes are electrophoresed into this precipitate, however, it solublizes, so that behind the migrating ellipse of antigen the area between the two side lines is clear. As progressively more antigen is left behind in the side line precipitates, the central zone of free antigen and soluble complexes becomes narrower, and the two side lines gradually converge. As the last of the antigen is precipitating, the two side lines merge to form a sharp peak, the tip of the rocket, which will then remain stationary during subsequent electrophoresis. Due to the accumulation of larger amounts of ag-ab complexes in the peak area, the tip of the rocket is denser than the sides. (Examples of rockets with this classical configuration are shown in Fig. 4a.)

The height of the rocket peak, or more accurately the area under the peak, is proportional to the amount of antigen applied in the sample well and inversely related to the concentration of antibody in the gel layer. This relationship constitutes the basis for employing RI in the quantitative determination of proteins. In our applications of the method, up to 23 samples are run on a single plate, and the first four of these are conventionally used to generate a standard curve of rocket height versus a series of known antigen concentrations.

As in other forms of immunodiffusion and electrophoresis, the density of the precipitin line is primarily determined by the concentration of the antibody. Thus, by diluting antigen and antibody to an equal degree, one can obtain a constant rocket peak height with a corresponding decrease in precipitin density. To maximize the sensitivity of the test for the detection of antigen, as well as to conserve antibody, the lowest antibody concentration generating distinct rockets can be used.

An important variant of RI that is useful for analyzing complex antigen-antibody reactions is a two-dimensional electrophoretic procedure named crossed immunoelectrophoresis (CI) (Laurell. 1965). In CI the antigen mixture is first resolved by electrophoresis through an agarose gel in the absence of antibody. The gel layer outside the electrophoresis path is then removed and replaced by

gel containing antibody; electrophoresis is resumed with the gel plate turned 90° to the original orientation, and the separated proteins are driven into the antibody layer just as in RI. CI has the highest resolving power of the available immunodiffusion and electrophoretic methods, because all precipitin peaks are formed with optimum and maximum binding of antigen and antibody.

In both Ouchterlony double immunodiffusion and Grabar and Williams immunolectrophoresis, sharp lines of precipitate are produced only between reservoirs containing equivalence concentrations of antigen and antibody. In complex systems, it is unlikely that all the antigens and antibodies in a single experiment will meet this requirement; some bands will be sharp and others diffuse, therefore, and this can lead to an overall pattern of poor resolution. This is not a problem with CI, in which differing concentration relations are reflected in the time at which the rockets stop elongating, and not in the diffuseness of the precipitates.

A major limitation of RI and CI is that proteins whose isoelectric points fall within the range between pH 6.5 and 8 do not electrophorese rapidly enough relative to antibody to be quantified (Laurell and McKay, 1981). It is possible to modify the charges of these proteins (Gross and März, 1988), but denaturation or alteration of specificity could be a problem here. For such proteins, it may be advisable to turn to a diffusion-based method, such as Mancini's radial immunodiffusion (Mancini *et al.*, 1965) or the Oudin technique (Telfer and Williams, 1953). Satisfactory rockets can be formed by basic proteins, but these, of course, are formed toward the cathode.

We generally follow the procedures recommended by Laurell and McKay (1981) for both RI and CI, with only two modifications. 1) We use a Tris-citrate buffer, 0.025 M, pH 8.2, which gives good separation of hemolymph proteins of moths as well as of bovine and human serum proteins. 2) For both RI and CI, we routinely cast a 1.2 mm thick agarose layer on double-sized (75×50 mm) microscope slides and use a sample volume of 3 μl per well (2.5 mm, diameter).

CHARACTERIZATION OF A COMPLEX ANTISERUM

For any quantitative immunochemical methods, it is desirable that a monospecific antiserum be used. However, when an antiserum contains antibodies to incidental contaminants in the sample used for immunization, or when a multispecific antiserum has been raised on purpose, its complexity is best characterized by RI and, in particular, by CI. In the example described here we characterized an antiserum prepared against microvitellin (MVN), a small, 31 kilodalton yolk protein isolated from the eggs of the *Hyalophora cecropia* (Telfer and Pan, 1988). The object was to obtain an antiserum suitable for quantitative RI of MVN in eggs and of its hemolymph precursor, microvitellogenin (MVG). The protein

proved, however, to be a poor antigen relative to other yolk proteins; in the course of generating a rabbit serum with a useful concentration of MVN antibodies, significant amounts of antibodies were produced to several contaminants that had not been visible by SDS-PAGE in the immunizing preparation. These became apparent when a 1% agarose layer containing 3.5% antiserum was reacted in RI with samples of haemolymph and yolk. The antiserum concentration was high enough to reveal relatively low concentrations of contaminating antibodies. MVG is found only in the blood of pharate adults and in yolk; it is not present in pupal blood (Telfer *et al.*, 1981). Ovariectomized pharate adult female blood and a yolk extract, along with a sample of pupal male blood serving as a control, were therefore chosen for the test. The resulting RI patterns are shown in Fig. 1a. The antiserum was clearly not monospecific: it produced two dense rockets with pharate adult female blood and yolk, and there were also three small rockets of very low density inside the major peaks. The minor rockets were also produced

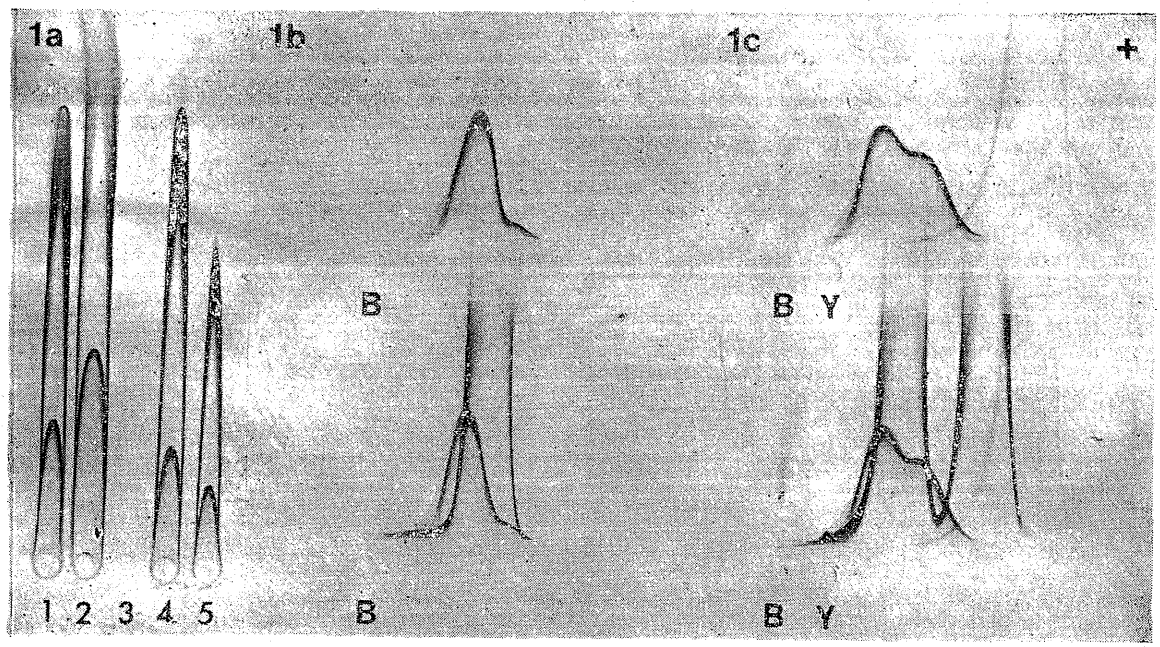


Fig. 1. Characterization of an antiserum against microvitellin (MVN) of *Hyalophora cecropia*. 1a. Rocket immunoelectrophoresis (RI) employing 3.5% (v/v) of the antiserum. Wells 1 and 2 contained 1/80 and 1/40 dilutions of a pharate ovariectomized adult blood, respectively. Well 3 had a 1/100 dilution of a *Hyalophora* male pupal blood, and wells 4 and 5 contained 1/40 and 1/80 dilutions, respectively, of a yolk extract. 1b. Crossed immunoelectrophoresis of pharate adult female blood (B) (1/40 dilution) used for RI. The first dimension run was at 8.7 V/cm for 40 min.. The second dimension was run at the same voltage for 3 hours. Top portion of the gel contained 3.5% antiserum which had been absorbed with pupal female blood; the bottom portion had 3.5% unabsorbed antiserum. 1c. Condition and gel composition was identical to 1b, except a yolk sample (Y) (1/40 dilution) was run in tandem with the pharate adult female sample (B) (1/40 dilution). The sample wells were 5 mm apart center-to-center. 3 μl sample volume was used in all plates.

by the pupal male sample. In addition, with the yolk sample, there was a very faint and fuzzy cap on top of the highest rocket, representing possibly another contaminant. Six different yolk and blood antigens were therefore detected by this antiserum. Isolates of MVN and vitellogenin (VG) (Telfer, 1954) were used to establish that the shorter of the two dense rockets, was produced by the former, while the taller dense rocket was produced by the latter (experiments not shown). These conclusions are consistent with other observations, such as the failure of pupal hemolymph to form the short rocket, and of male hemolymph to produce the taller one.

The reaction was greatly simplified by absorbing the antiserum with pupal female blood prior to its incorporation into agarose. (For more about the process of absorption, see below as well as Ma and Ballarino, 1988). Pharate adult female hemolymph now produced a single dense rocket with the height of the shorter dense rocket seen in Fig. 1a. The absorbed antiserum also retained an ability to form a dense rocket with isolated MVN (Fig. 2a), and this effectively identified the pharate adult blood reaction as due to MVG. Absorption therefore rendered the antiserum monospecific in its reaction with blood, and usable for RI determinations of MVG and MVN concentrations.

The reactions of this antiserum have also been analyzed utilizing CI. The lower gel in Fig. 1b, illustrates the results when ovariectomized pharate adult female blood was reacted with MVN antiserum at the same reagent concentrations as in Fig. 1a, well 2. The dense MVG and VG rockets were again formed, but they were now slightly displaced from each other, due to the faster electrophoretic mobility of VG. As with the RI tests, the taller, VG rocket was not formed when the antiserum had been absorbed with pupal female blood (Fig. 1b, upper gel). Fig. 1c shows that CI can be modified to yield information on the crossreactivity of antigens. To demonstrate this, a sample of yolk extract was electrophoresed along with but 5 mm to the right of the blood sample. Both of the dense rockets formed by yolk fused completely with their counterparts produced by blood, indicating that this hyperimmune serum was unable to distinguish between the blood and yolk forms of the two proteins. CI had thus confirmed the antigenic identity of VG and VN, as well as of MVG and MVN. (By contrast, we have shown that lipophorin, another major component of lepidopteran yolk, loses a set of epitopes when it is transferred from the blood into the oocyte (Telfer and Pan, 1988)).

The contaminant producing the fuzzy rocket close to the tip of the VN rocket during RI of yolk extracts, is probably due to the follicle cell product, paravitellin (PVN). Absorbing the antiserum with pupal female blood did not prevent the production of this band in CI (Fig. 1c, upper gel). Furthermore, it

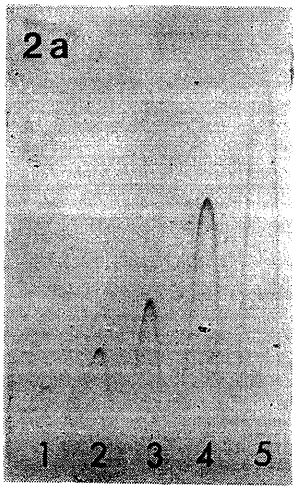
was never detected when blood from ovariectomized pharate adult females was added to the sample well, so this yolk protein is not detectably derived from the blood. It was more definitely identified as PVN by the fact that fused rockets were produced when isolated PVN and a yolk sample were analyzed by CI in tandem (data not shown).

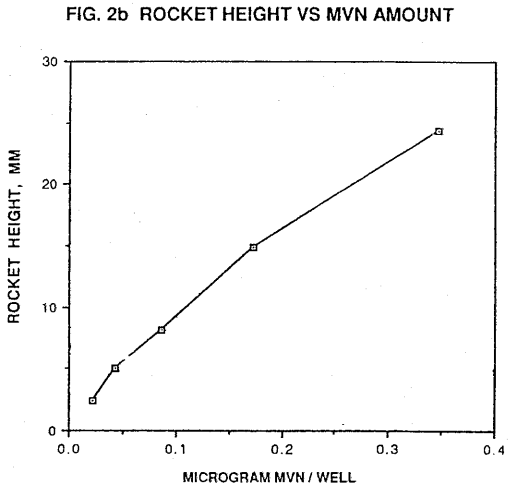
The most unique contribution of CI to the analysis of this antiserum was its demonstration that MVG and MVN can occur in two electrophorectically and antigenically distinct forms. Fig. 1b shows that the MVG in the blood sample analyzed produced a very small shoulder on the anodal side of the main precipitin peak. That this was due to a second form of MVG was verified later with other samples, which proved to have more of the electrophoretically fast form. Interestingly, where the two rockets fused, they formed a spur of precipitate, indicating that the fast form lacks a set of epitopes that are contained by the slow form (Telfer and Pan, 1988). Although in an earlier study (Telfer *et al.*, 1981), the existence of the two electrophoretic forms of MVG was noted, the antiserum used for that analysis did not reveal the difference in antibody binding specificity. To be certain of detecting differences of this sort, therefore, one must remember that the immune systems of different animals respond differentially to the same antigen molecule; to maximize the number of epitopes on a protein that are being examined, therefore, it is important to hyperimmunize several hosts.

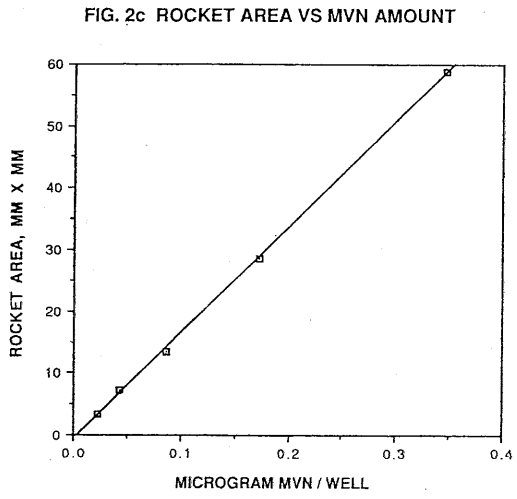
We conclude this section with some general comments on the use of RI and CI in the analysis of complex antisera. 1) In a multispecific antiserum, if the density of the precipitin line of the antigen to be quantified is distinctly greater than the others, it may not be necessary to go though an absorption process to eliminate the undesirable antibodies, for the rocket will stand out among others and be readily recognized. Or, one can simply lower the overall antibody concentration in the gel until the precipitin lines of the contaminating antibodies and antigens become invisible. 2) If known, isolated antigens are available, they can be used systematically to identify the different antibody constituents in the antiserum (Axelsen and Bock, 1972) and can then be used to absorb the antiserum to achieve the desired antibody specificity. 3) If an antiserum cannot be properly absorbed due to lack of the appropriate antigens, one can use CI to do the quantification, although it won't be as convenient and economic in terms of consumption of the antiserum. 4) The dilution of sample to run in CI is best determined by running a series of dilutions in RI first; a slightly larger concentration of antigen should be used in CI, as the antigen will be spread out during the first dimensional run and consequently the height of the precipition peak formed later will be low.

QUANTITATIVE DETERMINATIONS

A prerequisite of using any immunochemical methods to quantify proteins is that the protein standards used to construct the calibration curve and the assayed proteins must have identical antibody binding property. This can be ascertained by comparing them in a typical setup of Ouchterlony's double immunodiffusion, or with fused RI (Axelsen *et al.*, 1973). Alternatively, analyze them with tandem CI as shown above with MVG and VG (Fig. 1c). For quantification in RI, the antibody concentration in the agarose gel should be low enough to conserve the antiserum, yet high enough to produce well-defined rockets; this is simply a







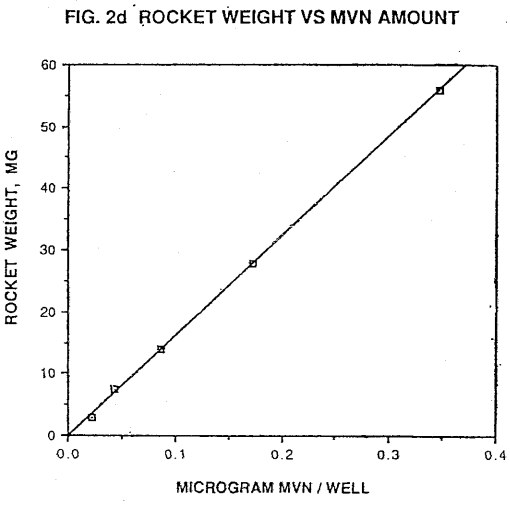
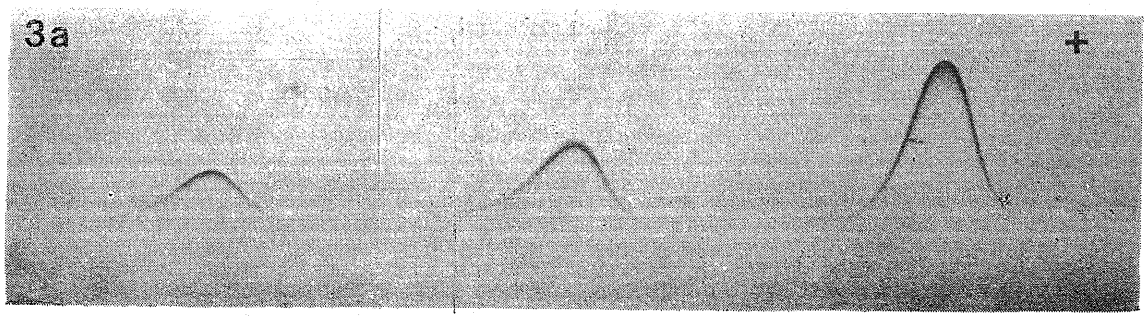


Fig. 2. Calibration curves for microvitellin (MVN). 2a. RI pattern of MVN standard; wells 1-5 had 0.022, 0.043, 0.087, 0.173 and 0.347 μg MVN, respectively. 2b. Heights of the rockets are plotted against the amounts of MVN. 2c. Areas of the rockets are plotted against the amounts of MVN. 2d. Weights of enlarged (1.53×) rocket cutouts are plotted against the amounts of MVN. All values were averages of two determinations.

matter of trial and error; try 1 to 3% antiserum concentrations to start out with. Low antibody concentration will maximize the difference of rocket heights produced by smaller amounts of proteins. Ideally, the samples to be assayed will have peak heights falling within the range established by the calibrating standards, and this can be found out only by testing several dilutions of the sample.

For the measurement of antigen concentration, one can use either rocket height or area. Gross and März (1988) reported that for some antigens rocket heights are not proportional to antigen concentration, and recommended that rocket area be used instead. We show here that this is true for MVN in our system. Fig. 2 shows three different plots of the RI pattern of MVN presented



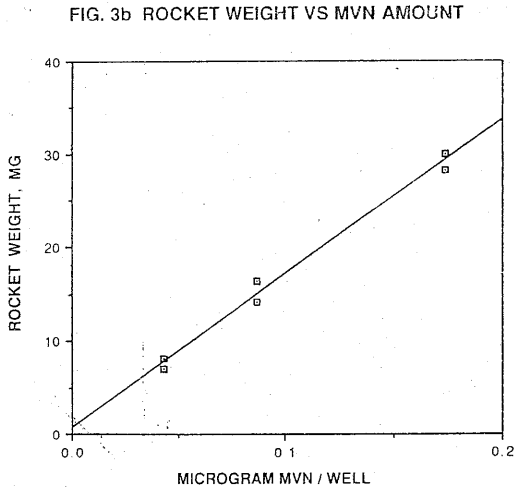


Fig. 3. Crossed immunoelectrophoresis of MVN. Two sets of three different amounts of MVN (0.043, 0.086 and 0.172 μg) were each run on a different gel plate. Antiserum concentration in the gel was 2.5%. Electrophoretic condition was similar to that in Fig. 1b, except the first dimension run was 30 min.. 3a. Precipitin pattern. 3b. Calibration curve. Weights of the enlarged (1.69×) peaks were plotted against amounts of MVN.

in Fig. 2a. Fig. 2b indicates that height is not a good measurement of MVN amount for a poor linearity is exhibited. However, in both Fig. 2c and 2d, an excellent correlation (r=0.999) between rocket area or weight and amount of MVN was demonstrated. The area of the rocket was estimated by taking the product of peak height times the width of the peak at half height. For the weight measurement, an enlarged copy of the gel plate was first made on a Xerox machine and pasted on a photographic mounting board to add some weight to the paper; the rockets were then cut out and weighed. (Alternatively, an enlarged photographic print of the rockets may be used.)

As discussed earlier, CI is also suited for quantitative work. For a demonstration, three dilutions of MVN were tested with CI (Fig. 3a). Since their peaks were not perfectly bell shaped, the weights were taken to establish the calibration curve, which turned out to be satisfactory (r=0.998) (Fig. 3b).

When rocket peaks are narrow, height measurement can then be used conveniently, as shown by the calibration curve for a sample of VG (r=0.999) (Fig. 4a and 4b). In this case, the value of the lowest amount of VG was discarded, because the antigen just barely electrophoresed outside the sample well and an accurate measurement could not be made. The difference in rocket shape between VG and MVN is unquestionably due to the differences in diffusion rate between 31 and 510 kilodalton proteins, which leads to greater lateral diffusion during electrophoresis in the case of MVN.

Based on the calibration curve in Fig. 4b, we determined the effect of repeated freezing and thawing of VG (Telfer and Pan, 1988). Aliquots of freshly prepared yolk extract were each subjected to a different number of freezing and thawing cycles; afterwards, several dilutions of them were made and their VG amounts were compared along with the VG standard in duplicate plates. The amounts of VG determined in each dilution were converted back to a value in the original yolk extract and an average was taken. The initial extract had a VG concentration of $9.73 \pm 0.50\ \mu g/\mu l$ (mean $\pm$ s.d., N=3) and the three sequentially frozen-thawed aliquots had values of 9.79 ± 0.47, 9.39 ± 0.67 and $9.59 \pm 0.26\ \mu g/\mu l$, respectively. There were no statistical differences between the values. Both the ability of VG to withstand the limited numbers of freezing-thawing and the accuracy of rocket measurements were demonstrated by this experiment.

For this experiment, we also deliberately used a rather high concentration of antibody (3% antiserum in the gel) to avoid having to make excessive dilutions of the yolk extracts; this was to minimize pipetting errors introduced in making the dilutions. In spite of this consideration, the samples still had to be diluted 1/300, due to their high VG content. Oudin's technique, with which sample

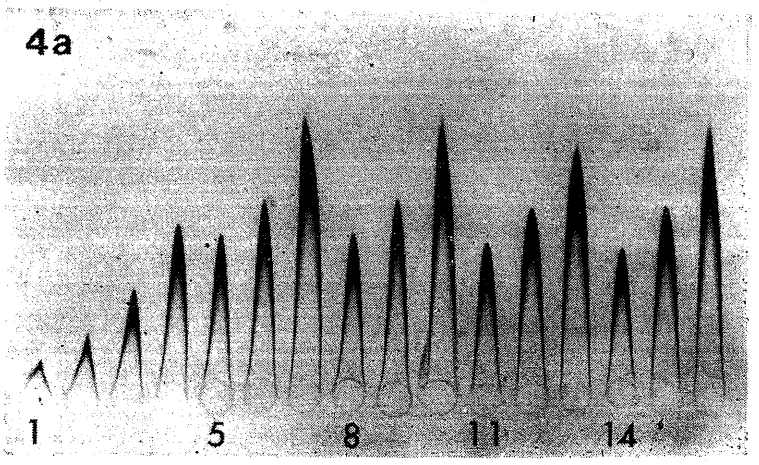


FIG. 4b ROCKET HEIGHT VS VG AMOUNT

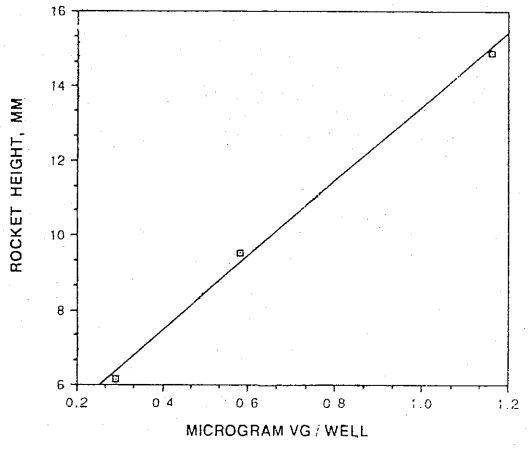


Fig. 4. Determination of the effect of repeated freezing and thawing on *Hyalophora cecropia* vitellogenin by rocket immunoelectrophoresis. The gel contained 3% antiserum to *Hyalophora* VG. 4a. Wells 1-4 contained isolated VG standard: 0.145, 0.290, 0.581 and 1.163 μg/well. Wells 5-7 had freshly homogenized yolk extracts: 3 μl/well of 1/200, 1/150 and 1/100 dilutions of the extract. Wells 8-10, 11-13 and 14-16 contained identical volumes of the same dilution sequence of extracts which had been frozen and thawed once, twice and three times, respectively. Another plate with extract dilutions of 1/300, 1/200 and 1/150 was simultaneously run (plate not shown). Averaged determinations were used for computation. 4b. Calibration curve of the VG standards. Heights of the enlarged (1.69×) rokets from 2 plates were measured, then averaged and served as the dependent variables. The peak produced by well 1 was not included in the computing (see text).

dilution is not usually needed, avoids this potential source of error. Notice also that much greater amounts of VG were used for the calibration curve (Fig. 4b) than in the case of MVN (Fig. 2). The few examples presented here are sufficient to demonstrate the range and flexibility of RI as a protein quantification tool.

IMMUNOCROSSREACTIONS OF THREE VITELLOGENINS

We introduce next an application of RI to the measurement of antigenic differences between homologous proteins from different species. Among several immunochemical methods employed for this purpose, microcomplement fixation (MCF), being the most sensitive, has been particular useful; a good correlation between immunological distance measured by MCF technique and percent amino acid sequence difference was noted (Wilson *et al.*, 1977). Beverley and Wilson (1982) constructed an evolutionary tree for 7 larval haemolymph proteins of *Drosophila* and two other dipteran species based on the immunological distances determined by MCF studies. Ouchterlony's double diffusion analysis has also been used for this purpose (Goodman and Moore, 1971) (see also Fig. 5); though the size and angle of divergence of the precipitin spur reflects the degree of immunocrossreactivity of the two antigens, precise quantification by this means is doubtful (Prager *et al.*, 1976). Nevertheless, it is a convenient way to gain some preliminary information. Axelsen and Bock (1973) employed RI and CI to examine the spurs formed by crossreacting human and bovine albumins, and found that both methods have higher resolving power than the double diffusion analysis. We report here a new way to use RI in studying immunocrossreactivity. Like MCF, our method yields a quantitative measure of crossreactivity. While it uses larger amounts of antibody, it has the usual advantage that RI offers in not requiring that the antigens be pure or the antiserum monospecific.

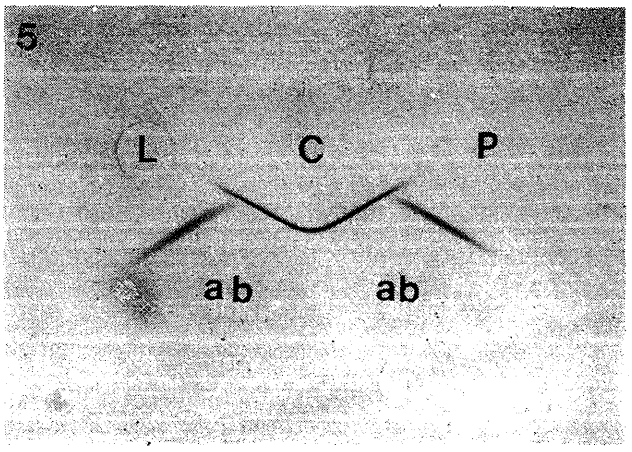


Fig. 5. Immunocrossreactions of vitellogenins of *Hyalopora cecropia* (C), *Actias luna* (L) and *Antheraea polyphemus* (P) in Ouchterlony plate. Antiserum (ab) was to VG of *Hyalophora*. Pupal female blood of each species was used for the test. Fusion of the bands indicates that the three vitellogenins have immunological similaritis. However, the spur formed at each fusion point indicates that *Hyalophora* VG includes epitopes that are missing from *Actias* and *Antheraea*. Since the degree of deviation and length of the spurs are similar, this test did not distinguish which related VG is more similar to that of *Hyalophora*.

The principle behind the method is simple. A given amount of an antigen will yield a higher rocket peak in an antiserum that has been absorbed by a crossreacting antigen than in the unabsorbed antiserum. In effect, the overall concentration of the antibody to the antigen used for immunization is diluted in the absorbed antiserum. The method yields a quantitative measure of similarity because the number and similarity of common epitopes determine the amount of antibody that will be precipitated by the absorbing antigen. Thus, the concentration of antibodies remaining in the antiserum after absorption is inversely related to the degree of similarity between the absorbing and immunizing proteins. Absorption with a distantly related antigen will reduce the residual antibody concentration to a small degree, and closely related antigens will cause a large reduction. Thus, by comparing the rocket heights after absorption with different antigens, one can compare their crossreactivities: the higher the peak the closer is the relationship. Bear in mind also that the density of the precipitin line is inversely related to the concentration of antibody, so that the absorbed antiserum which giving rise to the highest peak will yield the lightest precipitin line.

A major advantage of RI in studying cross reactions in that purified antigens are not needed; the only requirement is that there must be enough antigens available to absorb the antisera. Nor does the exact amount of the immunizing antigen used in RI need to be known. We describe here how the immunocrossreactivity of VGs from three species of saturniid moths was measured. The antiserum was prepared in a rabbit to the VG of *Hyalophora cecropia* (Telfer and Pan, 1988). Two aliquots were absorbed with samples of female pupal blood, one from *Actias luna* and the other from *Antheraea polyphemus*. The blood samples used for absorption had been compared with *Hyalophora* blood in an Ouchterlony plate (Fig. 5); band fusion with spur formation indicated in both cases the presence of a crossreacting VG, but the two spurs were so similar that it was impossible to distinguish any difference in the crossreactivities of the two related proteins.

To find out the amount of the pupal blood needed to absorb the antiserum completely, serial dilutions of female pupal blood of the related moths were tested in the Ouchterlony plates with the antiserum to *Hyalophora* VG (Fig. 6). The antigen dilution that gave the sharpest precipitin line, 1/20 in the case of this particular sample of *Antheraea* blood, was presumed to contain an approximate optimal proportion of VG. To ensure complete absorption, we used a 1/10 dilution: 1 part undiluted *Antheraea* blood added to 10 parts of antiserum (in the Ouchterlony plate, the volume ratio of antigen and antibody used was one to one) and a standard procedure of liquid precipitin test was followed. The antigen-antibody precipitate was removed by centrifugation; after this process, there would be small amounts of soluble ag-ab complexes left in the absorbed antiserum,

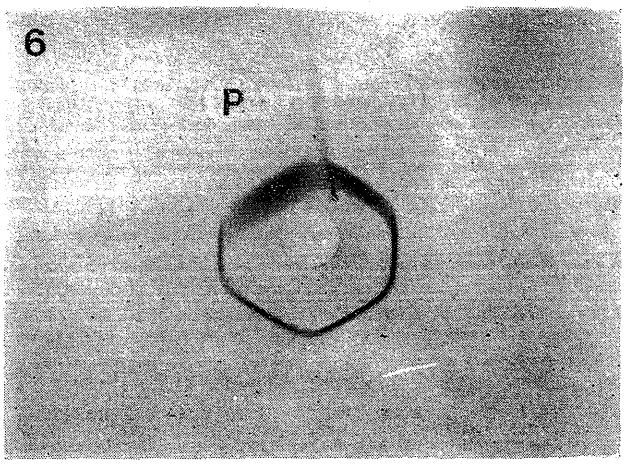


Fig. 6. A dilution series of pupal female blood of *Antheraea polyphemus* was tested against an antiserum to *Hyalophora cecropia* vitellogenin in an Ouchterlony plate. Center well: antiserum; peripheral wells clockwise from top left (P): 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160 dilution of *Antheraea* blood. 1/20 dilution gave the sharpest precipitin line, indicating that it approximated the optimal proportion of ag-ab binding. 1/5 and 1/10 dilutions clearly were in the antigen excess zone, the resulting precipitin bands were wide and diffuse. Each well was inoculated with 18 μl of sample.

but they should not interfere the analysis. In the RI test, it is critical that the absorbed and unabsorbed antisera be diluted to the same extent. To achieve this, one part of saline was added to ten parts of an aliquot of the unabsorbed antiserum. Other methods of absorption, such as affinity column chromatography, can not be employed unless there is a way of normalizing the absorbed antibody concentration in this way.

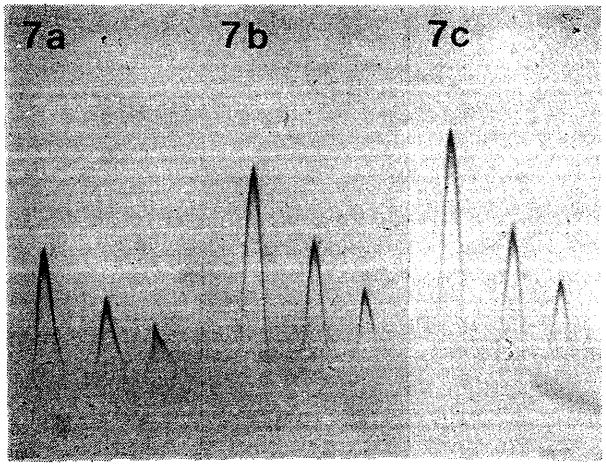


Fig. 7. Rocket immunoelectrophoretic patterns of a dilution series (1/75, 1/150 and 1/300) of a sample of *Hyalophora cecropia* pupal female blood run on a gel containing an antiserum to the VG of *Hyalophora* (7a) and gels containing the same antiserum previously absorbed by pupal female blood of either *Actias luna* (7b) or *Antheraea polyphemus* (7c).

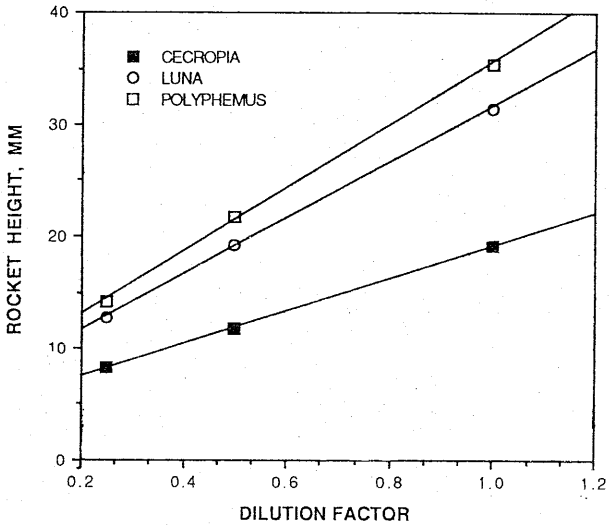


Fig. 8. Regression analysis of the data presented in Table 1. Rocket heights were measured from enlarged (1.66×) copies of the plates shown in Fig. 7. The differences indicate that *Antheraea* VG is more similar to *Hyalophora* VG than is *Actias* VG, so far as their reactions with this antiserum is concerned.

Table 1

Immunocrossreactivity of three saturniid vitellogenins (VGs) analyzed by rocket immunoelectrophoresis. The higher the value of crossreactivity is, the closer the immunological relationship. A sample of pupal female *Hyalophora cecropia* blood was tested with an antiserum to *Hyalophora*'s VG and two other aliquots of the same antiserum, one absorbed with *Actias luna* pupal female blood, the other absorbed with *Antheraea polyphemus* pupal female blood. The sample volume was 3 μl/well.

Antiserum	Sample dilution factor*	Rocket height, mm**	Slope#	C/L or C/P	Immuno-crossreactivity= (1−C/L or C/P)×100%
Unabsorbed	1.00	19.20	14.64= C		
	0.50	11.75			
		8.25			
Actias-absorbed	1.00	31.50	25.04= L	0.58	42% (*Actias*' VG to *Hyalophora*'s)
	0.50	19.25			
	0.25	12.65			
Antheraea-absorbed	1.00	35.40	28.19= P	0.52	48% (*Antheraea*'s VG to *Hyalophora*'s)
	0.50	21.75			
	0.25	14.15			

* Arbitrary; actual sample dilutions were 1/75, 1/150 and 1/300.

** Averaged measurements made on 1.66× enlarged copies of plates (Fig. 7) which were run in duplicate.

Slopes of regression lines of Fig. 8.

Since the effective antibody concentration in the absorbed antisera is lowered, a trial RI run must be made to find out the optimal antibody concentration to use in the gel. Again, use the lowest possible antibody concentration to give a visible sharp precipitin peak. This optimal antibody concentration should be used in all the RI plates for the final analysis. In our study of VG crossreactions, 100 μl of each antiserum was mixed with 5 ml 1% agarose. The RI pattern of the very same three dilutions of a sample of female *Hyalophora* pupal blood in the unabsorbed antiserum, *Actias* absorbed, and *Antheraea* absorbed antisera are shown in Fig. 7a-c. We could have used just one dilution of the *Hyalophora* blood to do the test, but three dilutions permitted us to do a regression analysis of the data, thereby yielding a better estimate. Fig. 8 shows the regression plots of rocket heights as a function of antigen dilution factors. Table 1 summarizes the results. The higher the slope of the regression line is, the closer is the antigen used to absorb the antiserum related to *Hyalophora* VG. We used the following equation to express the immunocrossreactivity or relationship: Immunocrossreactivity (ICR)=(1−(The slope of regression line obtained from unabsorbed antiserum÷ Slope of regression line obtained from absorbed antiserum))×100%. The larger the calculated value is, the higher is the crossreactivity or the closer the immunological relationship. From the data, *Antheraea*'s VG is evidently more closlely related to *Hyalophora*'s (ICR=48%) than is *Actias*' (ICR=42%).

A fuller analysis of immunocrossreactivity with this or any other method involved entail antibodies from more than one rabbit, reciprocal analysis of antibodies against the other species being studied, and as many species as possible in the taxon being examined. At this preliminary stage, there is also need for a comparison of different techniques, using the same antigen and antibody preparations. The utility of RI is nevertheless apparent, especially since it would facilitate studies where isolated antigens are not available, and where the antisera employed are not monospecific.

CONCLUSION

The applications of RI and CI include primarily the resolution of multiple antigen-antibody reactions in complex antisera, the measurement of antigen concentrations, even in situations in which the antiserum is not monospecific, and finally, the quantitative measurement of crossreactions. The advantages include ease of operation, minimum requirement of equipment, speed and accuracy of analysis (see Gross and März, 1988) and small sample volumes. In special situations other methods are more convenient and should be adopted. For versa-

tility and decisiveness, however, RI and CI are unparalleled and should be included in the technical repertoire of any laboratory using antibodies to study the behavior and functions of soluble proteins in animals.

ACKNOWLEDGEMENTS

This work was supported by a grant from NIH (GM-32909). We thank Peter C. Kulakosky for the MVN and PVN isolates.

REFERENCES

Axelsen, N.H. and E. Bock (1972) Identification and quantitation of antigens and antibodies by means of quantitative immunoelectrophoresis. A survey of methods. *J. Immunol. Methods*, **1**: 109-121.

Axelsen, H.H. and E. Bock (1973) Further studies on the reaction of partial identity in quantitative immunoelectrophoretic patterns. *Immunol. Methods*, **2**: 393-401.

Axelsen, N.H., J. Kroll and B. Weeke (eds.) (1973) A Manual of Quantitative Immunoelectrophoresis. *Scand. J. Immunol.*, 2, Suppl. 1.

Beverley, S.M. and A.C. Wilson (1982) Molecular evolution in *Drosophila* and higher Diptera. I. Micro-complement fixation studies of larval hemolymph protein. *J. Mol. Evol.*, **18**: 251-264.

Davis, E.M. (1988) Protein assays: a review of common techniques. *Am. Biotech. Lab.*, **6**: 28-37.

Goodman, M. and G.W. Moore (1971) Immunodiffusion systematics of the primates. I. The Catarrhini. *Syst. Zool.*, **20**: 19-62.

Gross, G. and W. März (1988) Immunoelectrophoretic techniques in protein analysis and quantitation. *Am. Biotech. Lab.*, **6**: 6-19.

Kulakosky, P.C. and W.H. Telfer (1987) Selective endocytosis, in vitro, by ovarian follicles from *Hyalophora cecropia*. *Insect Biochem.*, **17**: 845-858.

Laurell, C.-B. (1965) Antigen-antibody crossed electrophoresis. *Anal. Biochem.*, **10**: 358-361.

Laurell, C.-B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.*, **15**: 45-52.

Laurell, C.-B. and McKay, E.J. (1981) Electroimmunoassay. In: Langone, J. and H. Van Vunakis (eds.) *Methods in Enzymology*, vol. 73, Academic Press. pp. 339-369.

Ma, M.C. and J. Ballarino (1988) Immunochemical approaches to insect vitellogenesis research. *Sympo. Insect Biochem. Physiol.*, Taiwan, 11-23.

Mancini, G., A.O. Carbonara and J.F. Heremans (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, **2**: 235-254.

Prager, E.M., D.P. Fowler and A.C. Wilson (1976) Rates of evolution in conifers (Pinaceae). *Evolution*, **30**: 637-648.

Telfer, W.H. (1954) Immunological studies of insect metamorphosis. II. The role of a sex-limited blood protein in egg formation by the cecropia silkworm. *J. Gen. Physiol.*, **37**: 539-558.

Telfer, W.H. and M.L. Pan (1988) Adsorptive endocytosis of vitellogenin, lipophorin, and microvitellogenin during yolk formation in *Hyalophora*. *Arch. Insect Biochem. Physiol.*, **9**: 339-355.

Telfer, W.H., E. Rubenstein and M.L. Pan (1981) How the ovary makes yolk in *Hyalophora*. In: M. Kloza, eds., *Regulation of Insect Development and Behavior*. Scientific Papers of the Institute of Organic and Physical Chemistry, Wroclaw Technical University, pp. 637-654.

Telfer, W.H. and C.M. Williams (1953) Immunological studies of insect metamorphosis. I. Qualitative and quantitative changes in the blood proteins of the cecropia silkworm. *J. Gen. Physiol.*, **36**: 389-413.

Wilson, A.C., S.S. Carlson and T.J. White (1977) Biochemical evolution. *Ann. Rev. Biochem.*, **46**: 573-639.