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his very interesting work with acatalasemia and galactosemia. Then, I want to go from this area to my own work on glucose-6-phosphate-dehydrogenase in cell culture.

I would like to call on Dr. Herzenberg to begin with his comments on antigenic markers in cell culture.

Study of the H-2 Locus in Murine Cell Cultures

by Leonard A. Herzenberg

Herzenberg: I might say that most of this has been given in a formal presentation a few months ago* at the Gatlinburg (Oak Ridge) symposium (41), so I don't think a formal repeat of this would be at all in order. I would just like to start the discussion. I don't think we have to keep it at all formal as far as I'm concerned. I think everybody should pop in, as they have been doing.

[Slide] We have here an impressive chart, impressive because you won't be able to understand it, and anything we can't understand is impressive, or, perhaps, repressive.

Koprowski: Could we have the lights out?

Herzenberg: Yes, but you're not going to see much more of it, I'm afraid.

Koprowski: You're right. [Laughter]

Szybalski: Is this Egyptian or Assyrian?

Herzenberg: What I have up here, actually, is a chart summarizing the work that has gone into the immunogenetics of the H-2 locus, or the H-2 chromosome region in the mouse.† This covers a period of some twenty-odd years now, involving a large number of people, especially George Snell and Jack Stimpfing at Bar Harbor, the late Peter Gorer, Bernard Amos, Gustavo Hoeker in Chile, and others.

The H-2 (Histocompatibility-2) locus in the mouse, which really could be just called an isoantigenic locus, determines antigenic differences between individuals which can be detected by other individuals

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† A revised nomenclature for H-2 antigens is now in preparation by George Snell and others and will undoubtedly be published.

in the same species. One of the ways in which this has been studied is by the grafting of tissues; therefore, histocompatibility. It could just as well be called a blood group locus, because it determines antigens located on the red blood cells.

There are a large number of alleles, certainly in excess of 26, at this point which have been found in different inbred mouse strains, or, in some cases, in individual mice, which have then been bred to get strains. Some of these may have arisen by crossovers (for example H-2^5) between some of the existing alleles. The allele is, of necessity, somewhat inexact.

Let me describe this in general. The H-2 locus is actually a region of the chromosome that has been found to have crossing-over to the extent of about 1 percent in the mouse, which is a very high level. Therefore, it is a large region of the chromosome. This locus determines a series of distinguishable antigenic components, distinguishable on serological bases, in general. These antigenic components are described by letters, which were in the body of the table, and, on the right-hand side, we have the particular strains in which these were found.

The matter of interest to us here is, first, if these antigens could be shown to be maintained on cells growing in culture indefinitely, they could be used as genetic markers. These would be true genetic markers in the sense that the characteristics that we can examine will be determined by a typical Mendelizing unit in the mouse. We have shown that, indeed, these antigens persist indefinitely, in culture on all cells we have looked at. The oldest cells that we have looked at are now six years old. The H-2 antigens are present as well on the C3H cells that Earle started in 1940.

The question now is, how can we detect these antigens on individual cells, so that we can do true genetics in culture? A variety of methods present themselves immediately, and several of these have been worked with and explored, with reasonable degrees of success. A detailed report has been published (10, 11).

If one has a cell which is appropriately sensitive to the killing action or cytotoxic action of antibodies in the presence of complement, one can detect the antigen on the cells by the fact that the cells are killed. It is perfectly straightforward. It turns out that cells of lymphoid origin are most sensitive to the cytotoxic action of antibodies and complement. We have obtained a number of antigenically marked cell lines in culture (or cell strains—I'm not sure which), which are indefinitely propagatable at this point. Is it a strain?

Gartler: No, it is a line.

Herzenberg: They are killed by appropriate antibody and complement.

The efficiency of killing with ML-388—we have worked with this line for the longest period—is about 99 percent, under the best conditions.

Koprowski: What did you mean by "lymphoid origin?"

Herzenberg: In this case, this is from a methylcholanthrene-induced lymphoma. Originally, it was a histocytic cell, according to the pathologists, and then it became more of a macrocytic-looking cell. Is that right? At this point, if you make a Wright stain of it, it has a large nucleus and a little bit of cytoplasm around it.

Eagle: How many discrete antigens are you dealing with in a specific type of culture?

Herzenberg: In this particular case, we have been dealing with a cell that arose from a homozygous mouse, a mouse homozygous for the H-2^a allele. We have shown that the killing is exclusively, or almost exclusively, due to antisera directed against antigens determined by the H-2 alleles. There are lots of other antigenic differences between these cells and other cells, but the killing is specifically due to H-2. We can show this in several different ways.

Now, within the H-2 complex of antigenic components, we have been able to get killing with so-called monospecific sera. I say "so-called," because all this means is that by serologic means, sera have been fractionated by absorption or by suitable immunization and then absorption, so as to react with only one *known* component.

It has been the history of all immunogenetic investigations that the more you look at a single component, the more you can break it up into a large number of components. What the antigen or antigenic component in this case is, at the molecular level, I wouldn't care to say. I don't think we can really come to any conclusions on it at this point. There are always the two conflicting possibilities: one, that an antigen component is a specific region of a molecule, and the other, that it is a reflection of the complexity of the serum which is used to detect it; in other words, there is a whole series of different antibodies, and the antigenic component is, in a sense, defined by the antibody which reacts with it. I don't know if I am just confusing us more, but the point is that we cannot make any conclusions at the molecular level as to what we mean by an antigenic component at this point.

Szybalski: Does this 99 percent efficiency mean that if you plate a hundred cells, you get one colony which is growing?

Herzenberg: It means that if you have a hundred colonies growing, without selection, you have one colony growing with selection.

Szybalski: Yes, and this one colony is now different, or is it just a survivor?

Herzenberg: No. I'll get to that. It is not different as yet.

Ephrussi: Excuse me, but what is the cloning efficiency?

Herzenberg: It varies between 30 and 50 percent.

Steinberg: An H-2 allele produces more than one known antigenic factor. Is the killing due to only one of these, or do you require more than one of them, or is one more efficient than the others?

Herzenberg: It is determined by more than one antigenic component. You can get killing with one or two or several. I am not prepared to say what the relative efficiency of killing is with the various ones, because what you mean by having antibodies against a particular component is that you take a complex serum and fractionate it, or else you prepare sera in different mouse strains and you cannot directly compare a particular antibody in one strain with another.

McKusick: What is known about the chemical nature of the antigen? Did I understand that this is a lipoprotein in nature rather than a polysaccharide?

Herzenberg: The evidence at this point is not at all compelling. Everybody has jumped on the bandwagon of assuming that this antigen is a lipoprotein.

All that can be stated with certainty is that the antigenic activity, determined by the inhibition of hemagglutination (in other words, the absorption of hemagglutinating antibodies), the induction of hemagglutinins, or, based upon another serologic test, the enhancement phenomenon in mice, is located in a lipoprotein-rich fraction. It is just a shift of orthodoxy, without any real basis. I might say I may be responsible for this, because we have published the first paper (42) in which we found it was a lipoprotein fraction, and we did not conclude it was lipoprotein, by any means. Everybody else has now jumped onto this (38).

We are dealing with an antigen which we really don't know anything about as to its chemical structure. We know very little about the molecular configuration, antigenic components, or antigenic factors, if you wish, which are determined by this region of chromosome in the mouse. Nevertheless, we can detect the antigens on cells in culture, and one more very significant thing, I think, is that in either meiotic situations, or particularly in meiotic situations, crossing over or genetic recombination between these various components has been observed in a reasonable number of cases at this point, probably about a dozen,

and a crude linkage map is being developed, particularly by Stimpfling at Bar Harbor, at present.

This is based upon the very naive, but possibly correct, and, I think, only possible kind of hypothesis, that the arrangement of these components can be made in linear fashion; that is, perhaps, there is some direct relationship between the genetic map and the antigenic components here. I say this is naive because we have no information about the molecular structure of the antigen, and there may be a series of steps between the gene product and the antigen in this case. One cannot, with justification, make a direct analogy between, say, the structure of hemoglobin and the structure of the gene, and the problem here.

Now, we say we have a selective system, so far, with respect to these antigens in culture, which is not a very highly efficient process. After one passage of this sort, where we pick up one colony out of a hundred which grows after selection, still in this case it has the original antigenic composition. If we carry this selection over 14 passages, somewhere between the eleventh and the fourteenth passage, we end up with a mixture of cells, some of which are no longer killable by serum and complement and others of which are still as sensitive as those we started with.

Eagle: Excuse me, Leonard, but I am a little puzzled here. If you are dealing with a multiplicity of antigens, all involved at the same H-2 locus, is your selection done with an antiserum to one of this complex of antigens, or to the entire complex?

Herzenberg: It is with an antiserum directed against a number of the components, neither all of them nor one. It is against, in this case—it is an antiserum prepared by injection of tissues. I should, perhaps, have said this in the beginning. All the antisera we are working with are sera prepared by injection of tissues from one mouse strain into another. They are never prepared against the cells that are cultured. We are dealing with antigens which are exactly equivalent to the antigens found on the cells in the original animal, as far as the reaction with antibodies at least is concerned.

The antisera in this case are all prepared in one mouse strain that carries the H-2^b allele, against tissues from mice carrying the H-2^a allele, and we have made use of the coisogenic strains of Snell, which differ genetically only by the allele, or essentially only by the allele, carried at this particular locus, the H-2 locus. They are genetically identical, within practical limits, except for the H-2 locus. These sera,

therefore, are relatively clean with respect to antibodies against antigens other than H-2.

We are not selecting, therefore, against a single antigenic component, certainly, at this point. As I said, after 11 to 14 repassages with constant selection, we ended up with a mixture, a mixed population of cells, resistant and nonresistant to the cytotoxic action of serum and complement. These were then cloned out. We cloned many times before that, looking for this, but at that point they were cloned out, and we did end up with stable cultures of cells resistant to the killing action of the antiserum and complement. We have a number of parameters by which the sensitive and resistant cells are different. However, the resistant cells do still have the H-2^d antigen, but in lesser amount per cell.

This is not a contaminating cell because of that fact alone. These cells will still form a tumor when inoculated into a mouse of the H-2^d phenotype. They have not gained as far as we know an antigenic difference, which prevents them from growing in animals having the H-2^d genotype.

Ephrussi: Excuse me, but it seems to me that there is good evidence that the antigenicity controlled by this locus in quantitative terms, does decrease in particular, with ploidy.

Herzenberg: Yes.

Ephrussi: Are these by any chance cells with higher ploidy?

Herzenberg: We have looked at them, and they are not grossly different in ploidy. We have not counted individual chromosomes, but we have not gone from a diploid or near-diploid to a near-tetraploid.

Gartler: Does the resistant line clone as well as the parent line?

Herzenberg: They clone with about the same efficiency. There are a number of things which are different about them. They grow a little more slowly. The cells are larger. The amount of antigen per cell surface area is about one-quarter to one-eighth as much. They have this difference in morphology, I think, in both clonal and individual cells, in gross appearance (11).

Eagle: How do you interpret their resistance now to the antigen?

Herzenberg: I would probably interpret it on the same basis as Möller (72) interprets the relative resistance; I mean Möller in Klein's laboratory—on the same basis as he interprets the resistance of sarcomas, or the relative resistance of sarcomas and carcinomas relative to lymphomas, that there is less antigen surface area, and you don't get as much complement bound which lyses the cells, but this interpretation is not relevant, I think, to genetics specifically.

I might say I admit that this was simply a model which we had available for seeing whether any of the methodology might work. Briefly, other ways of picking up the antigens on the cells with which we are working in mass cultures are absorptions or neutralization of antisera with these cells (10). If the antigens are present on the cells, we can immunize the mice with these cells and, by appropriate genetic control, we can show which antigens are there. These are rather crude methods and not very useful for genetic purposes. A promising non-selective technique is labeling the cells with fluorescent antibody methods. We can label these cells by reacting them first with mouse antiserum and then with fluorescein-conjugated rabbit antimouse-gamma-globulin.

You get very nice ring reactions, provided you keep the cells alive. This technique, again, has been described by Möller (73) for tumor cells and tissue cells, and it works reasonably well for culture cells. I say "reasonably well." It is not perfect.

It is essential that the cells be kept alive for the staining to be specific and clear, and it is just this property which we want to have here, that you don't kill cells unless you add complement. The antiserum alone is not toxic. We are hoping, therefore, although we have not yet done this, to pick up stained and unstained cells, or small clones, and see what sort of differences we have here.

But now, the main object of our tooling up—and this has been a long tooling-up procedure, of preparing all sorts of different mouse antisera in sufficient quantities and getting a number of different cell lines growing in culture—our main interest now is to work with cells derived from F-1 hybrids or from heterozygous animals, in the same way as the Kleins have been working with tumors *in vivo* in mice, selecting for losses of particular antigens or antigens determined by particular alleles in mice. We wish to do this in culture.

Let me just say why. The first cells which we have available, which we could grow, as I said, were homozygous, to start out with. This is if they were diploid. If they were aneuploid in culture, we don't know how many doses of this d allele we have in the culture cells. We may have two or three or maybe even more. Conceivably, we could have one, but I think this would be less likely.

If we start out with a cell, however, which is heterozygous, that is, H-2^d over something else, then we can select against H-2^d, and we will need only a single change to find the loss of the antigen. This is what Klein has done in the mouse *in vivo*, and he has been very successful in showing that with the so-called heterozygous tumors, he

can get loss of the antigens, determined by one or the other of the alleles in the heterozygote, by grafting the tumors back into one of the parental strains which made the original heterozygote. You can put such a tumor back into a mouse which has the H-2^s homozygote state, and the tumor will not grow. These cells will not grow in such an individual but will be rejected by the homograft reaction, unless a change to loss of H-2^d occurs in some of the cells, and, indeed, this happens all the time in his material.

An H-2 allele determines a whole series of antigenic components. In the mouse, using the homograft reaction, it is not possible to dissect these components very far. He can, in one particular case, dissect the H-2^a allele into essentially what is d and k, and show loss of either k alone or d+k in such a situation. He has interpreted this as possibly being due to mitotic crossing over in these cells.

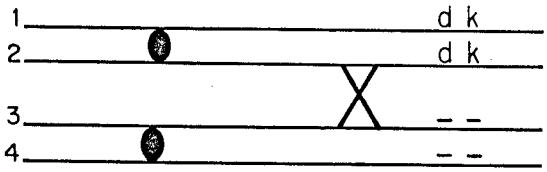

Figure 27. See text.

For example, let's consider a diploid cell which is heterozygous, we will say, in this case for d and k, arranged in this order along the chromosome (Fig. 27). If mitotic crossing over occurs between chromatids 2 and 3, and then chromatids 2 and 4 go to the same pole at segregation, that cell will have lost both d and k. If, however, crossing over of the same chromatids were to occur but *between* d and k, then the cell will have lost k but not d. In order to lose d without losing k, you have to have a double crossover. Since Klein finds loss of only k or loss of d and k, and never loss of d alone, he favors the hypothesis that the loss is due to somatic crossing over.

Atwood: What is the relative frequency of loss of both to loss of k?

Herzenberg: They are not very different from each other.

Atwood: Well, then, it must mean that either the hypothesis is wrong or d and k are very close to the centromere. [Laughter]

Herzenberg: Yes. There is another possibility; that is—and I suppose that we ought to be sophisticated enough to realize that double crossing over in small regions is not very rare at all. It seems to be an

increased frequency of crossing over, very close to another crossover, a sort of negative interference.

This is purely an interpretation, and we are far from the actual possibility of proving it at this point. But, in culture now, if, indeed, there is some sort of linear arrangement of the antigenic components, we might have, say, the antigenic components, A, B, C, and D, in a given cell, in the heterozygous state. Our intention is to select with an antiserum directed against one or just a few of these components, and, hopefully, detect the true variants or true losses in progeny cells, and then check to see what other nonselective antigenic components are lost at the same time. This is, again, simply a hope for the future, and, perhaps, for a little more immediate future than it was two years ago, but there is no experimental evidence to add on it at this point.

We now have at least two of Klein's lymphomas growing as lymphocytic cells in culture, growing in suspension, unfortunately; they do not attach to the glass. They are nice, round cells, and you put them back in the animal and they form a tumor, and you put them back in the culture and they grow in culture. They still maintain their heterozygous antigens. They have not been selected at all. But cloning is difficult with them because they do not attach to the glass.

Bayreuther: Can't you overlay them with agar?

Herzenberg: I am sure there are all sorts of tricks one could apply. But, every time you try to apply a new trick in culture, it takes you six months to get it to work properly.

Stern: How far are d and k on the meiotic crossing over map? Is that known?

Herzenberg: There are twelve crossovers or so known at this point, six of them published and six of them just told to me. The statement cannot be made with any certainty. The whole region is said to undergo about 1 percent crossing over.

Stern: But there is no special reason to believe that d and k are extremely far away?

Herzenberg: These have been shown to be crossovers.

Atwood: There is one point that might be relevant to this. If these come from crossovers, then, the allele that was with them has become homozygous, probably, and this means that it would be lost, then, less frequently than it was lost before, when it was with the original d and k. Is that true or is it not?

Herzenberg: In Klein's experiments?

Atwood: Yes. I wondered if you had looked for that.

Herzenberg: You mean, the allele that is with them should be homozygous in another cell?

Atwood: The cell that has lost both d and k at once by crossing over should be a homozygote for the other allele.

Herzenberg: For s, yes.

Atwood: Yes, for s, and then it would be lost much less frequently than s is lost in the original heterozygote.

Herzenberg: Yes. In fact, if they should start with a cell derived from homozygous mice, then, they have never selected a true loss variant. They can select false-positives, as they call them, but in all cases these have turned out not to be true loss variants.

Atwood: Well, then, that makes this test even better.

Herzenberg: But I don't know if they have looked at these kinds of—yes, it would make the test better, but whether they have actually looked there, I don't know. I don't think they have published on them.

Neel: Can we go back to Dr. Stern's point, that if, already, there are some twelve crossovers between d and k, and if the chromosomal region involved in these antigenic properties may extend some distance beyond your markers then the actual region involved may have considerably greater potentialities for crossing over, if you test for the right antigens.

Herzenberg: No, there weren't 12 crossovers with d and k. There have been some 12 crossovers all together between different components in the H-2 system. With this information, it is becoming possible to assign a unique sequence of the components. It is a tremendous job to look for crossovers here. What you have to do is to set up an F-1 cross between two individuals, and then test the progeny of the cross with a whole battery of antisera. The mouse hemagglutination system is not the most ideal system to work with, so they have to try them at a variety of antisera concentrations and be absolutely sure of their results in each case.

Neel: But is it fair to say that most of these crossovers have been discovered accidentally rather than systematically?

Herzenberg: The first one was discovered accidentally, but Stimpfling is now looking for them systematically in two particular crosses.

Neel: So the frequency of recombination might be considerably higher than the 12 known cases to date would imply?

Herzenberg: Not considerably.

Renwick: In Klein's work, in the surviving cells which are, presumably, homozygotes for 2^s—has he tested for dosage factors? That's the same point Dr. Atwood was mentioning.

Herzenberg: Yes. They published on this (55), trying to reach the conclusion that it is not hemizygous but is homozygous; that it does have more antigen than the heterozygous cell does. I have had a lot of experience with mouse serology, particularly using the hemagglutination method which they have to use, and I would be very loath, and I am sure they would agree, to accept these conclusions completely; that it is very difficult to tell a twofold difference in the amount of antigen per cell, because it is just a twofold dilution, you see, and everything is done by serial twofold dilutions, so the difference between whether you see a weak agglutination or you see none in the final tube of the agglutination series—

Renwick: As far as it goes, it is consistent with homozygosity, is it?

Herzenberg: Yes, it is consistent with it.

DeMars: Do your selective methods or typing methods actually lyse the cells, or do they just become rounded and sit there and fail to divide, or what happens in your culture?

Herzenberg: In some cases you can see them lysing, but not all of them.

DeMars: Is it quickly?

Herzenberg: Yes, they lyse within ten to fifteen minutes.

DeMars: That's too bad.

Motulsky: Is there any change in the titer or the level of antigen in the primary cultures, as compared with when they become permanent, or have you tested this at those stages?

Herzenberg: There isn't a very large difference between the amount of antigen on the cells that have been in culture for six years and those put back into an animal that grows the tumor, when you look at the tumor cells. There is not much difference, comparing the amount of antigen on these ML-388 cells that have been in culture for six years and another tumor arising from the same animal, at this time.

Koprowski: But you actually do not have primary cultures of normal origin. We are only discussing cells of tumor origin.

Herzenberg: Well, we have primary cultures of normal origin, bu they are not terribly useful because we can't kill them, so far. W have them, and they stain with fluorescent antibodies, but our primar aim at this moment is to select differences.

Motulsky: But you say all of these lines, if you let them sit, becom permanent type?

Herzenberg: Yes.

Motulsky: When they become permanent, do they then show t properties of being lysed by the antiserum-complement system?

Herzenberg: The two that we have, that are round cells deriving from these lymphomatous lines, do. They are still sensitive. But fibroblasts are not sensitive.

Motulsky: How many different H-2 alleles have you worked with at the cell-culture level so far? How many have you taken?

Herzenberg: Four, but one is a sort of combination of the other two; that is, a combination of d and k, so it would be three.

Szybalski: Does this mean that you have four independent selective systems at this time, or how many selective genetic systems do you have?

Herzenberg: One is no good at all. The anti-b is not a good selective system.

Sybalski: How many good selective systems do you have?

Herzenberg: Three, but, as I say, again, one of them is not very good. Actually, there is a multiplicity, when you really mention it, because the allele d determines a series of antigenic components, and we have shown that we can kill with fractionated sera, with sera directed against just one or a few of these components; so that it is just a matter of how many different fractions of sera we can make. But you can function—

Szybalski: What is the selective efficiency of this system? About 1 percent? Did I understand you correctly?

Herzenberg: In one passage?

Szybalski: Yes, but I am thinking more along the experimental designs as normally employed in microbial genetics. Do you know anything about spontaneous mutation rates in this system?

Herzenberg: As far as antigenic loss goes?

Szybalski: Yes.

Herzenberg: Well, we have been working. At this point, we have data on these things only with the homozygous cell, and we have found none, as you notice, that have actually lost the antigen. There is a decrease in amount, which we ascribe to—

Szybalski: Does this mean that the cells have mutated and have changed to heterozygotes from homozygotes?

Herzenberg: I don't think so. The cell type has changed. It is probably just a fairly large change, and may be due to a single kind of primary change which we have no idea what it is, but it results in these cells which are different in several ways, as I showed you at the beginning, and are resistant to the killing action of the serum. But they are not true antigenic losses, which is what we want to work with.

Szybalski: What I am really asking is whether, at present, your system is ready for real *quantitative* type of genetic experiments?

Herzenberg: It is practical for one kind of experiment right now, and that is what we are doing now; that is, starting with heterozygous tumors, selecting against just one of the alleles, a whole allele, which is the most sensitive system, and seeing if we can't get a complete loss of antigen in the same way that Klein has gotten in the mouse. I'm just following on his coattails.

DeMars: The nicest way to nail down mitotic recombination here would be if you could find the twin spot. In the culture environment where the cells are fixed on the glass one can work as with Drosophila. This is the reason for my original question. If you start with cells that have double heterozygosity in repulsion $(\frac{a+}{+b})$ one sort of segregation following recombination would yield the $\frac{a+}{a+}$ and $\frac{+b}{+b}$ homozygotes. These segregants would form colonies side by side and each colony would have lost one of the antigens if enough growth had occurred following segregation. If you select against the antigen of one cell type then the reciprocal segregant would survive. Furthermore, if the selective treatment is such that the cells selected against are morphologically altered but not killed then both cell types might be recoverable and subject to further examination.

For instance, could you grade your selective treatments, so that, instead of lysing the cells, they just became rounded or sick-looking? This would allow you to scan the culture and find the patches of cells that look perfectly fine.

Herzenberg: Bob, this is what we intend to do with the fluorescent antibody, to allow small clones to develop, and stain them. They are not killed by that. We know this. They are not killed by just antibody, once we leave out the complement. You can look at them without killing them.

DeMars: Fine. Then you will be able to look for twin spots.

Szybalski: Would their frequency be reasonably high for this type of experiment?

Herzenberg: I don't know. Based on Klein's material, it is, yes.

Neel: Before we leave the material which has just been presented, I wonder if I could ask Dr. Herzenberg about the kind of problems that he thinks can be attacked with this system of his.

Herzenberg: Well, the first one is the question of whether gene processes or genetic processes can, indeed, be studied in somatic cells

in culture; that is, can you find true gene mutations in culture? Can you find mitotic crossing over in culture? Perhaps, we don't have to question any longer whether it is possible to get exchange of genetic material from one cell to another. One has to develop markers for this and ways of studying the markers in individual cells. Is this satisfactory for the record?

Renwick: Wouldn't you add segregation in general, not just somatic crossing over?

Herzenberg: Yes

Neel: Could you distinguish between mutation and somatic crossing over?

Herzenberg: On paper, yes, very easily. [Laughter]

Neel: I wasn't thinking of the paper demonstration.

Herzenberg: As yet, we have no evidence of either occurring for sure in culture, so I don't see how we can distinguish between these things —I mean, experimentally, between things which we have not found yet. No, I don't mean to be facetious, but if you were to find, for example, selecting against one of the antigenic components here, others obligatorily changing at the same time in some cells, and then you selected for another one and other ones changed at the same time, you might, if it was really always the same kind of change and you couldn't build up any kind of a linear sequence on the basis of this, then you might expect all these changes were mutational changes.

If, on the other hand, you found that it was easier to build up the linear sequence from the changes that build up, you might suspect that these were due to somatic crossing over or mitotic crossing over. You could look for the twin spots, as Dr. DeMars has suggested; that is, you should look for the reciprocal type. If you find it, then, this would prove that you had mitotic crossing over.

DeMars: I think it is important here to keep in mind two things. First, in order to establish a sequence with mitotic recombination, several markers on the same arm of a chromosome will be required. This will take many more markers than are now available and lots of time before they become available.

That is why, in my own thinking about this, I have assumed that we were going to have just one marker to work with. You can formally distinguish between nondisjunction, mutation, haploidization, and mitotic recombination in a rigorous way using just one marker, provided that you rig the scheme so that you can recover the twin spot.

Second, it is not enough even to find the reciprocal crossover type in a population. If you can show that the two reciprocal types arise

side by side, and if you can make a chromosomal analysis of the two segregants and rule out nondisjunction, you can also rule out mutation and haploidization. That is why I asked the question concerning your ability to detect and characterize the reciprocal type when you select against one of the serologic types.

Herzenberg: You have two kinds of twin spots.

DeMars: You can have two kinds of twin spots, as Dr. Stern pointed out, and one marker is enough to give you the twin spots that you need.

Neel: But, with the amount of migration in these cultures, if there are "twin products," are they going to stay side by side?

DeMars: You were seeing a greatly magnified microscopic view, but on a macroscopic scale, they would be close enough.

Ephrussi: Twin spots (in the genetic sense) are the obligatory result of the process being explained here. But there is no necessity at all that there should be geographical localization, as there is on the integuments of Drosophila.

DeMars: Well, they don't have to be like that [clasping hands]; they can be like this [intertwining fingers]. How are we going to get that in the record? [Laughter]

Chu: There are two facts that may be helpful to supplement Dr. DeMars' comments. The first point is that all the mouse chromosomes are acrocentrics. So we have only one arm to worry about. The second is that with the knowledge of sex-linked antigens in the mouse I guess one can make use of the hemizygous state.

Herzenberg: They are not usable yet.

Chu: Well, I was just asking the question.

Herzenberg: They were found just a few months ago, by very long graft rejection; I mean, the only evidence for it is the rejection of grafts, after thirty or forty days, I believe.

Renwick: How do you distinguish between mutation and haploidization with one marker? I don't think you can (except by chromosome studies), unless you have a marker like the H-2 complex.

DeMars: If it is haploid, I don't suppose you can, very readily. I would be very happy to find either one. But you can certainly make a good formal demonstration of mitotic recombination in this material, provided you have the proper selective scheme.

Renwick: If you have a locus such as Dr. Herzenberg is working with, you have effectively got multiple loci to work with; that is, he has so many mutant sites on the one locus that he has the equivalent of a large number of loci to play with.

Ephrussi: I would like to point out that sometimes it does help to see things, and while we speak of twin spots, I think that our chances of detecting them in a visual manner would be greatly enhanced by the use of epithelial tissues. That is where you have a slight chance of seeing really geographically separated twin spots. That is one thing.

The other thing to which I would like to call your attention is something much less sophisticated, and therefore less fashionable, namely pigmentation. Pigmentation would be a very good visual marker; in fact, we are planning to try the visual detection of hybridization by using two strains of mouse iris, which have no pigment for different genetic reasons, so that cell fusion (which we are interested in) would result in the appearance of pigmented spots on a colorless background.

Neel: I might say that, some years ago, I looked for a somewhat different kind of twin spot, namely, to see whether in a Negro known to be a carrier of the gene for albinism, to see if we could find twin spots of albino and somewhat darker tissue, because there is some evidence that the albinism gene in the Negro is a diluter of pigment. It was a forlorn hope.

Gartler: I thought we might spend a few minutes on the work that has been carried out attempting to detect the ABO antigens in human cell culture. I don't know if anybody here has worked with these markers at the cell culture level and if not I will briefly review this area. Has anybody here worked with the ABO markers in cell culture?

There are three interesting papers on this subject (43, 51, 56). Högman (43) reported that he could detect the ABO antigens in the first cultures that grew out of fetal kidney and lung tissue explants. In the primary culture, roughly 50 percent of the cells exhibited a mixed cell agglutination reaction for the appropriate antigen. However, after a short period of time, the cells lost their reactivity. Apparently, only the epithelial cells in the culture exhibited any reaction, and as fibroblast-like cells overgrew the culture, all reactivity was lost. In this regard, it is of interest to note that Dr. Herzenberg mentioned that while his fibroblast cultures maintained antigenic specificity, they were not lysed in the presence of antiserum and complement as were the nonfibroblast cultures.

Herzenberg: I believe that is irrelevant because different cell types in the mouse are known to have different sensitivities to the cytotoxic action of sera, whether or not it is culture.

Gartler: Kodani (56) has just published a report essentially confirming Högman's work. That is, that the ABO antigens are detectable

early in the culture history and that they disappear after a short time in culture. He has made one additional observation of interest. Cells may pick up an antigenic specificity from the serum in which they are cultured: for example, O cells may exhibit a weak A reaction when cultured in A serum.

Renwick: The work which George Le Bouvier (63) did very much confirms this, along the same lines. He found, using mixed agglutination techniques, that there was a decline in the proportion of cells which showed the antigen among those that were sticking to the glass.*

As far as I know, he believes that most of the cells that showed the antigen were epithelial in type. These tests were all done on cultures derived from foreskin, and it is clear from some other work he did that the cultures can contain cells of different types, all looking similar, but having, for instance, different viral sensitivities. He has no evidence that a given cell or its clone loses its antigen, whereas he has evidence for artefactual selection against antigen-carrying cells during subculture, the selection arising when the medium, with its suspended cells, is discarded prior to trypsinization.

Gartler: The only other work I know of along this line is that of Kelus, Gurner, and Coombs (51) who, several years ago, typed the HeLa culture. The blood types of the individual from whom the HeLa cells were obtained are known. She was MN-positive, and they were able to show MN reactions, using rabbit anti-MN.

Renwick: I think it is important to point out here that he could not safely distinguish whether it was M or MN. It was an "MN-type antigen" (51).

Gartler: And I think her blood type was MN, and this was still present on the HeLa cell culture.

Renwick: I believe that isn't so. The MN group of the donor of the HeLa line is not known. Coombs has been misquoted, I am sure, many times. I don't think there are any of these blood groups which he did which were really specific in the sense of distinguishing between two different antigens of the same system. Coombs and his collaborators were able to show an MN type antigen; they were able to show H antigen which belongs to the ABO system, and they were able to show the Tj^a which is present in practically everybody and is

*Afterthought: He found that among the cells which did not stick, after trypsinization, or which came off the glass, the proportion of antigen-carrying cells was much larger. These were maintained in roller-tube cultures for up to three weeks, but did not establish themselves on the glass and eventually degenerated.

nonspecific. As far as I know, there is really no definite evidence that two antigens of a single system could be distinguished at that age of a tissue culture.

Herzenberg: Actually, I think that Dr. Gartler is correct here, that the patient was MN, and they did detect, with rabbit serum, M and N specificity. If there is any antigen which I bet would be more likely to be maintained for a long period, of the commonly known blood group antigens in man, it would be the MN.

Renwick: The MN grouping in red cells is pretty reliable, but MN grouping in tissues by mixed agglutination is not so specific (83).

Neel: Why would you bet that one would persist?

Herzenberg: Because I think that it is found on cell membranes of a variety of tissues. It is a messy one, in that M goes to N. But I think, and Coombs would agree with this, that although it is messy to work with, he has the strong impression, as the cytologists continue to insist, that the M and N are found in a variety of cell membranes throughout the organism.

My bet for kinds of antigens that you would want to find persisting in cultures would be those that fit the following requirements: one, that they be on white cells, generally speaking, and, two, that in species where you can work with the situation (and, perhaps, in man, you can do this as well), it be clearly histocompatibility antigens. In other words, you could induce isoantibodies against them.

Renwick: I think that the easiest antigens to work with would be those that you know are still present; in other words, use the long-term cultured cells as your antigen stimulus, and see what antiserum you can produce from those, instead of doing what has been previously done—looking in culture cells for antigens which you knew beforehand did exist in the individual.

Atwood: The genetic basis for this is known, whereas any antigens that you revealed newly on old lines are not known.

Renwick: But you can then go to the original family again and use the antiserum produced from your culture.

Herzenberg: What are the essential prerequisites? If you want to work with human antigens, you have to be willing to utilize human beings, with tissue cells. If you work with rabbits, I think, you're going to run into all sorts of difficulties.

Bayreuther: One system that has not been mentioned here is the polyoma virus induced cell antigen, discovered by Sjögren and Habel (92, 33). This new antigen is present in tumors induced by polyoma in mouse and hamster cells *in vivo* and *in vitro*. The genetic basis

for this new antigen is unknown. The antigen is exhibited in cells that do not produce any virus or any constituents of the virus. After longer periods of *in vitro* cultivation the antigen can be lost. These cells maintain, however, their neoplastic properties. This is a new field, just opening up. R. Bases has done some *in vitro* work in Dr. Dulbecco's laboratory. Applying techniques similar to those used by Herzenberg, Bases prepared antisera against cell membranes or whole tumor cells induced by polyoma. The presence of the new antigen—presumably surface—is revealed by the fact that the transformed cells are specifically sensitized to the killing action of complement by the antiserum. This killing is measured quantitatively by the loss of the colony-forming ability of single cell suspensions.

Ephrussi: Did you say that the polyoma antigen can get lost, without loss of neoplastic character?

Bayreuther: Yes.

Ephrussi: Is that loss of the antigen detected at the culture level or at the cell level?

Bayreuther: On the cell level. These cells can be cloned with high efficiency. Exposure to antiserum does not sensitize them to the action of complement.

Herzenberg: That doesn't mean necessarily they have lost the antigen, as we have found. They still have the antigen. They just have a little less of it.

Bayreuther: That is quite possible. Bases has different lines showing different degrees of loss. Some of them seem to have lost the antigen completely.

Eagle: As shown by what? By killing or fluorescent antibodies?

Bayreuther: By killing and fluorescence.

Eagle: Because Herzenberg's data show the fluorescent antibodies do it.

Ephrussi: Do you think the karyotypes correlate with these gradual losses, or what? Has this been looked for?

Bayreuther: That has not been looked for.

Eagle: If you put this antigen-free transformed cell into the animal and it forms a tumor, has the tumor regained the antigen?

Bayreuther: No, it does not. It seems not to induce immunity either.

Ephrussi: You mean, using it for rejection and then challenging the animal?

Bayreuther: It does not induce immunity. Tumors with the antigen are not rejected, when the animal had been previously challenged with a high number of killed cells free of the antigen.

Koprowski: Perhaps one should immunize against polyoma and then compare the results of challenging the animals with each of the two kinds of cells: cells with the antigen and cells without it.

Herzenberg: Isn't it true that the only evidence that the antigen is truly lost and not just inactivated biologically, in terms of the cell being allowed to be killed or rejected, is the fluorescent antibody? Is that conclusive?

Bayreuther: Yes, it is conclusive.

Herzenberg: I might just mention that, technically, we find that the fluorescent staining is certainly not an all-or-none process. About 5 percent of the cells from populations that are positive do not stain, but we cannot pick out these cells. They are not antigenically stainable. Their mixed progeny is stainable, again, which, I assume, is just some sort of a technical reason why these cells are not staining, an inability to demonstrate the phenotype.

Gartler: But, in this case [Bayreuther's], I assume, it is a population difference which fails to show the reaction, not just a small percentage of the cells; so this is quite different.

Ephrussi: Is there any correlation between strength of antigen-antibody reaction, in this case, or by tests, say, of your antiserum plus complement? In other words, is there a correlation between that and the growth phase of the culture, or the growth rate?

Herzenberg: No. We have looked for this, and we have not found any. I won't say there isn't any, but we have not been able to find it. We have not been able to find a particularly sensitive period or a particularly resistant period.

Eagle: Boris, why do you ask the question?

Ephrussi: Well, I am asking the question because I think there is very little systematic study of the different growth phases when, for example, claims are made that cells derived from a well-known organ necessarily lose their specific differentiation and enzymatic activity. Very little account is taken of the fact (or of the possibility, at least), well known in microorganism, that a number of enzymes really build up only during the stationary phase. For many reasons people work chiefly with the exponential phase, during which many enzyme systems are at the minimum.

I personally would expect that, during the exponential phase, you would find the cells to contain all the enzyme systems required for the process of cell duplication. However, when it comes to specific activities connected with differentiation, you would look into the nonubiquitous enzyme systems, and these may very well be elabor-

ated just in the stationary phase. In so far as the antigens are concerned, I don't know whether there is a cycle in the culture, too.

Renwick: An illustration of what you indicate is known in human cells. The quantity of cell-bound acid mucopolysaccaride is greatest in slowly growing cells, I believe (74).*

Ephrussi: This could be expected as well on the basis of altogether vague indications in the old tissue culture literature, but much better, say, on the basis of Gale's work on bacteria, which showed that there is a cycle of elaboration of a number of enzyme systems, which come up, really, at the end of a long growth cycle. I myself published a study about ten years ago on the synthesis of cytochromes in yeast, showing that the whole growth cycle, except the last two generations, goes on at the expense of glycolysis, and that cytochrome oxidase is elaborated only at the very end of the growth cycle, when growth slows down and switches from the exponential to the stationary phase.

Billingham: Do you get that sort of effect with your retinal pigmentation?

Ephrussi: Yes, very definitely, I think, because pigment formation is very clearly inversely proportional to the growth rate.

Eagle: Yet, this is not true for at least two of the lines which continue to function well in culture, those studied by Sato and Buonassisi (8). The reason I asked Klaus [Bayreuther] the question about what happens when you put it [polyoma-altered cells that have lost polyoma antigen] back in the animal is that their adrenal culture loses its capacity to respond to pituitary hormones after x months of culture. If these cultured cells are then reimplanted into the animal and produce a tumor, and that tumor is recultured, the capacity to respond to pituitary hormone is restored, as if the cell had somehow been recharged.

Herzenberg: Or, perhaps, the culture had been recharged.

Ephrussi: Exactly. In this case, you really don't know whether there is not some interaction with something coming from the organism into which we implant.

Herzenberg: Or selection. He had not ruled out selection.

Eagle: No, he has not ruled out selection. I asked him that specifically.

DeMars: It might be worth while, since we are immediately going to talk about biochemical defects of the cultured cells, to give some

*Afterthought: The *total* production of acid mucopolysaccharide, however, shows the opposite effect—it is maximal in rapidly growing cells (Morris, 74).

examples of what Dr. Ephrussi means, and I think that I can show two slides that will make the point.

We have been studying a number of enzymes in cultured cells, and we have been very prissy about it, in the sense of asking ourselves what an enzyme determination means with these cells. I will be very brief about this.

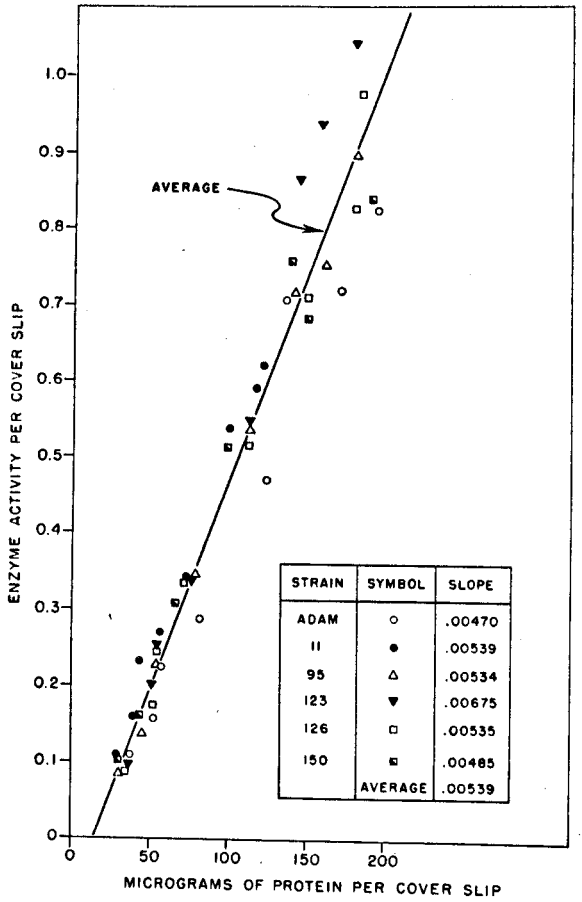

Figure 28. The appearance of acid phosphatase activity during the growth of six strains of diploid human cells. The graph relates total enzyme activity per culture to total cell protein per culture at different points during growth and shows that acid phosphatase is formed at constant specific activity throughout growth.

We took into account variations in enzyme activity during the culture cycle, and we have found so far two different patterns relating enzyme activity to growth during the propagation of the cultures. The first case involves acid phosphatase, which is almost always considered to be an ill-defined collection of enzymes. I think this may be unjustified in the case of the cultured human cells. In any case, it exemplifies an important rule.

These experiments are done by making a series of replicate cultures on coverslips and then assaying the enzyme activity directly on the dry monolayers. Samples of coverslips are taken daily for making determinations of enzyme activity and total cell protein.

Figure 28 shows one experiment with six different strains of cells: two normal male, two chromosome 18 trisomy, and two normal female. Each point represents duplicate determinations of enzyme activity and of total cell protein at a given stage of the culture cycle. The samples were taken daily.

You can see that for acid phosphatase, these points form a beautiful straight line, and the six strains represented here form a remarkably homogeneous group. We can make a ratio of total activity to total protein and define the enzyme specific activity.

In determining enzyme activity in this case, if you do the whole experiment and then derive the slope of the curve, you get one estimate of specific activity. If you do what people often do, which is to take the culture at some single, poorly defined stage, usually when it is pretty heavily grown, you can get pretty much the same estimate of specific activity.

Things are not as simple for beta-glucuronidase, which is shown in Figure 29. This is exactly the same kind of experiment. Mrs. Gorman in my laboratory has been studying this enzyme. Again, we have a variety of euploid and aneuploid strains. The same experiment is performed, with daily determinations of total enzyme activity and total protein. You can't make straight lines with these curves, and, in particular, in some experiments, when the cultures have gotten near the maximum density, you can get steep rises in total enzyme activity without a commensurate rise or, indeed, any net change in protein content of the culture.

In another experiment, many replicate cultures were made, and one set of them was handled in the usual way, which is having the medium replaced daily to provide the maximum growth rate. The other sets of replicate cultures were fed in, in this way, for zero days, for two days, four days, and then transferred to a medium that had been taken

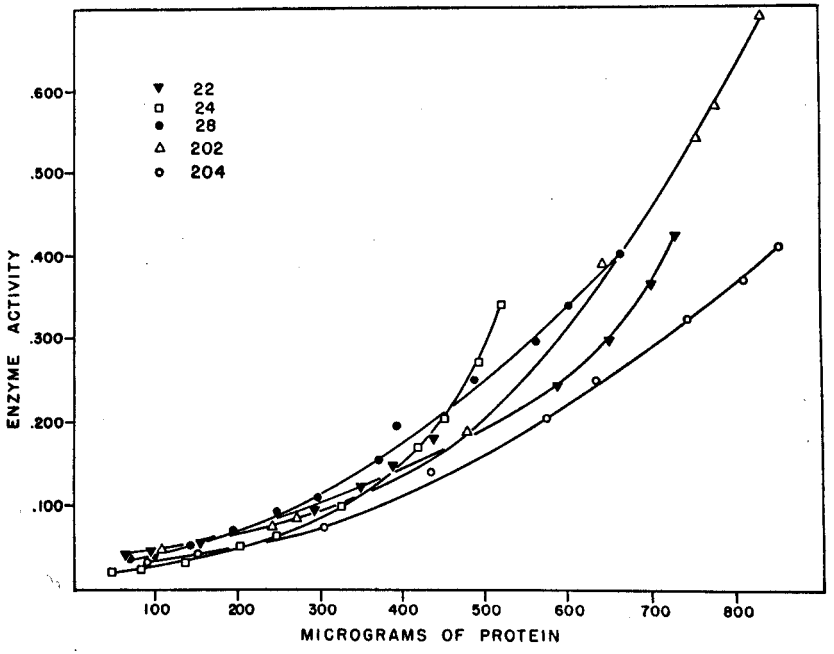

Figure 29. The appearance of beta-glucuronidase activity during the growth of five strains of diploid human cells. The experimental technique and manner of graphical presentation are as for the determinations of acid phosphatase described in Figure 28. These curves show that the specific activity of beta-glucuronidase increases significantly during growth of the cultures.

From Jean M. Marsh, and Robert DeMars, "Acid Phosphatase Activity in Cultivated Human Cells," *Experimental Cell Research* (1963).

from cultures of maximum population density, so-called used or conditioned medium. In such a medium the cells do not grow very well.

The cell protein showed almost no change in the used medium, but there was a steep rise in beta glucuronidase activity.

This is very different from acid phosphatase. With acid phosphatase, if you don't get a change in protein content, either a lowering or an increment, you do not get any change in acid phosphatase. Here, [in the case of beta glucuronidase] big changes in enzyme activity can accompany little or no change in total protein.

The other thing is that this will, of course, remind people of the work reported by Cox and Pontecorvo (14), working with cultures

derived mainly from foreskins. Alkaline phosphatase behaves very much in the same way in response to used medium, although I don't think they have published experiments of exactly this sort. But the formal aspects of the problem are the same, I think.

Mrs. Gorman has gone one step further. She has asked herself: "Does this represent the unmasking of protein that is already there but enzymatically inactive, or does this represent or depend on the synthesis of new protein?" She has used puromycin, which is a specific inhibitor of protein synthesis, and, under conditions where protein increase is blocked by puromycin, you cannot elicit these steep rises. You freeze the protein content and you freeze enzyme activity.

I think, therefore, in a general way, these are related to the remarks of Dr. Ephrussi, because, under the ordinary conditions of cultivation, beta-glucuronidase is found in substantial amounts only when the population has almost or actually stopped increasing. We have no idea of what beta glucuronidase is used for by the cells, nor do we have any idea what alkaline phosphatase is used for by the cells, but, in both cases, these enzymes are characteristic of the maximum stationary phase of the culture.

If you take a population like this, that has built itself up, and then subculture it, it does not maintain, or it does not start out with a high level. It is back where it started, and it goes through the whole process again. It is not a question of selection for high enzyme cells. It is a recurring cycle of low relative rate of enzyme formation and then higher relative rate of enzyme formation, conditioned in some way by what the cells do to the medium, apparently, or, maybe, what the medium does to the cells. We don't know.

Ephrussi: However, in the case of the cytochromes, we have shown rather clearly in our paper, I believe, that the cycle is an effect of glucose, and that, by decreasing the glucose, one can prevent the cycles from occurring.

DeMars: In this case, we are not having glucose repression; that is, if we lower the glucose content of the medium down as low as we can, beta glucuronidase still goes—

Ephrussi: I was not trying to suggest that it necessarily is glucose, but I mean it may be as simple as that with respect to some other constituent.

DeMars: Well, we always invoke glucose. If you have had experience with microbes, you always worry about glucose.

Ephrussi: Oh, sure.

DeMars: So we worry about it, too, and it isn't important here.

What seems to be important is that a variety of treatments that slow the growth of the cells boosts the relative rate of glucuronidase formation. For instance, lowering the concentration of serum in the culture medium slows the growth of the cells and accelerates the rate of glucuronidase formation. Lowering the glucose content slows the growth rate of the cells, but does not boost the glucuronidase formation.

Eagle: How about the omission of amino acid?

DeMars: We have not done this as yet.

Renwick: One question arises from Dr. DeMars' comments, which I know he has thought about. Is this effect of used medium a nonspecific increase of a large number of enzymes, due to some nonspecific effect on a certain batch of metabolic processes or is it due to the accumulation in the medium of, let's say, some substrate which induces these enzymes fairly specifically?

I want to make only one comment on the work of Cox and Pontecorvo (14). I looked at the electrophoretic mobility of the alkaline phosphatase in the induced cells, and all the bands were increased, roughly, tenfold in the same proportion, so, in the case of alkaline phosphatase, we can add a little bit more than just one enzyme to the total number of inducible enzymes.

DeMars: I think I might add some general comments. The different shapes of the curves that relate enzyme activity to growth in the cases of acid phosphatase and beta glucuronidase indicate that the definition of rather small differences in specific activity, differences as small as twofold, may require more than precise determinations at some arbitarily chosen, single point of the culture cycle. In addition, repeated determinations throughout the culture cycle may be necessary.

Now, Dr. Herzenberg asked me one question, which I did not take up at the time; that is, in the straight-line curves for acid phosphatase activity as a function of growth, the best straight lines fitted statistically almost never passed through the origin, that is, zero enzyme, zero protein. They always passed through the protein axis, in a way which can be interpreted in the following manner:

At the beginning of the growth of these cultures, that is, immediately after subculture, the cells act as if they had lost enzyme activity that was present just before subculture, without a commensurate loss of total cell protein.

Now, he is very familiar with this phenomenon, and I think that many people are now; that is, that the procedures we use in subculturing these cells often lead to losses of not only amino acids or

other metabolites of low molecular weight, but as well to losses at least of activity and probably of substance, of high-molecular substances, such as enzymes. In the case of acid phosphatase, these cells act as if half of the acid phosphatase activity were lost relative to the total protein content upon subculture. This happens with beta-glucuronidase, also. It is even more striking when one looks at the gamma glutamyl transferase of HeLa cells, for there, we can boost the activity to a maximum by growing them in the absence of glutamine. If we subculture cells with the maximum level of this glutamyl transferase enzyme, and then examine the enzyme activity relative to the total protein content of the inocula, as soon as possible after the cells have attached, which means within several hours, one finds that if one uses small inocula, one has lost as much as 90 to 95 percent of all of the enzyme activity that was present initially. Nevertheless, these small inocula grow very well and quickly recover their enzyme levels.

In the case of the enzyme in the HeLa cells, the loss of activity, or the apparent loss of activity, is a function of the size of the inoculum. The relative loss, therefore, is smaller, if you increase the size of the inoculum. But when one uses inocula so great that many of the cells fail to attach, one never recovers more than half of the activity that one puts in the bottle to begin with.

There is, then, another lesson here, I think; that is, in making enzyme determinations with these cells, there is a real premium on either avoiding the physical manipulation of the cells, either with scraping, centrifuging, or suspending them, trypsinizing them, and so on, or, if this cannot be avoided, then, one must have very reproducible procedures for manipulating the cells.

Chu: May I just ask a technical point? What kind of method do you use for subcultures—scraping or trypsinization?

DeMars: We use trypsin regularly.

Chu: Do you know about the effect of trypsin on cells?

DeMars: On enzymes, also. That is not a problem here. We can rule that out, that trypsin has inactivated the enzyme in the cells.

Gartler: I think we ought to go on to the next topic. Dr. Krooth will take over now.