AN ANALYSIS OF MITOSIS IN LIVER RESTORATION

By AUSTIN M. BRUES, M.D., AND BEULA B. MARBLE

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, Boston)

PLATE 1

(Received for publication, July 27, 1936)

Sections of liver tissue taken during the process of hypertrophy following partial hepatectomy show varying numbers of karyokinetic figures, at times even as great as those seen in rapidly growing malignant tumors. This fact has been noted by all who have studied this phase of experimental pathology. The chief object of this investigation has been to correlate mitosis in this tissue with the known growth rate.

Higgins and Anderson (1) have shown that when the main lobes of the liver of the white rat are removed, the remnant, which comprises about 30 per cent of the original liver, begins at once to hypertrophy. The rate of increase in size is greatest during the 1st day of hypertrophy, and restoration is complete after about 3 weeks. Brues, Drury and Brues (2) reinvestigated this matter from the point of view of cell restoration and showed that there is no increase in hepatic cells during the 1st day of restoration, and that cell restoration, throughout, lags about a day behind tissue restoration.

A number of questions in regard to mitosis in liver restoration have never been satisfactorily settled. There is still considerable discussion as to whether the predominant form of cell division in this organ is karyokinetic or amitotic. Some writers have thought that mitosis was more frequent in the periphery of the lobules than elsewhere, or in cells recently developed from budding bile ducts, while others have failed to make such observations. Moreover some writers have assumed that in this and other tissues, the number of mitoses seen at any given time can be considered as an index of the growth rate. We have sought to establish answers to such questions as these.

Method

Inbred albino rats of the Slonaker strain were used in these experiments. Animals were chosen which weighed between 100 and 180 gm. Partial hepatectomies were done by the method described in another paper from this laboratory (2), at which time it was demonstrated that the residual fragment left in the rat was 31.6 ± 1.5 per cent of the total liver.

Animals were sacrificed at various time intervals, some under ether and some by decapitation after stunning. The liver was removed as rapidly as possible and thin slices were fixed in Zenker's fluid with 5 per cent acetic acid, in every case within 5 minutes of the animal's death, and in most cases within 1 or 2 minutes. This precaution was taken since Thuringer (3) has shown that mitoses become progressively less numerous in the human prepuce as the length of time elapsing between operation and fixation of tissue increases. No differences in the appearance or frequency of mitoses were observed between the livers removed under ether and those which were not subjected to the anesthetic.

After dehydration and clearing, the tissues were embedded in paraffin and sections 6μ in thickness were cut, and stained with iron hematoxylin and eosin. A few sections, to be used for examination of mitotic spindles, were stained with phosphotungstic acid hematoxylin as described by Warren (4).

Sections in which chromosomes were to be examined were stained by the Feulgen reaction as described by Ludford (5) and counterstained with saturated picric acid.

In one series of animals in which it was desired to follow the incidence of mitosis over a period of time, biopsies were taken from the hypertrophying liver under brief ether anesthesia. The operative incision was reopened and a wedge-shaped snip of tissue between 1 and 2 mm. in size was cut from the edge of the organ. When successive bits were removed they were taken from widely separated places. Following biopsy in this fashion, there was no obvious reaction except locally in the affected area.

Mitoses were in every case counted under the oil immersion objective, and over an area containing at least 1000 hepatic cell nuclei. At least 400 nuclei were actually counted; and in sections of even thickness, other measured areas of the same size were assumed to contain (within the reasonable limits of error) the same number of cells. The incidence of mitosis is always expressed as per cent of hepatic cell nuclei dividing in a given area. Fig. 1 is given to show examples of the earliest and latest stages which were recorded as mitotic figures. Where no mitoses are recorded, it is to be understood that at least 2000 hepatic cells were counted. In many cases the phase of mitosis was noted, as prophase, metaphase, anaphase or telophase according to the judgment of a single observer. The greatest source of error here lies in the fact that some anaphases of which a small part was included in the section have doubtless been called metaphases. Since mitotic figures occupy, on the whole, a somewhat larger space than resting nuclei, it is likely that the absolute percentages of dividing cells found are actually a little high, but as all

counts were done on sections of the same thickness (6μ) this error should be the same in all cases.

RESULTS

An occasional mitosis (but never more than one in 10,000 to 20,000 cells) can be seen in resting liver. During the 1st day after partial hepatectomy few, if any, more can be seen. Near the end of this time, it can be seen that there is a very noteworthy variation in the size of nuclei, which are remarkably uniform in resting liver. This condition persists for the next 2 days, and then slowly becomes less striking. Although it is likely that the larger nuclei are most prone to divide, this cannot be observed directly.

No "abnormal mitoses" have been observed in our series. All hepatic cell mitoses in any given phase are about the same size. It has not been practicable to count the chromosomes in our material. Mitoses in the smaller bile duct cells are always much smaller than those in hepatic cells (see Fig. 2). No attempt has been made to count the mitoses in bile duct cells, but it seems that their incidence is roughly parallel to that of mitoses in hepatic cells, and likewise they are not seen during the 1st day.

On a small series of sections of actively dividing tissue stained by the phosphotungstic acid hematoxylin method, a few mitotic spindles in hepatic cells were chosen and drawn with the camera lucida, in order to estimate the angle of the spindle. In eight spindles conforming to the requirements laid down by Warren (4), that is, lying perpendicular to the line of vision, the angle was found to be between 60° and 70°, with an average value of 64°. This is a relatively narrow angled type of spindle. One of these spindles is shown in a photomicrograph (Fig. 3).

The question of the distribution of mitoses throughout the organ was investigated in a section removed 48 hours after operation. The mitotic incidence in this section was over 3 per cent. About one-half of a complete transverse section of a lobe was mapped out with the aid of a camera lucida and all the mitoses were drawn in place, and the phase of each was noted. The drawing was then divided into 300 equal squares, 379 mitoses were found, and the number of squares containing respectively no mitoses, one mitosis, and so on, was counted.

This number of squares (out of the 300) containing each finite number of mitoses is shown in Table I, together with the theoretical number of squares which should have the same number within. This calculation is done assuming an entirely random distribution, according to the

TABLE I

No. of mitoses	Actual No. of squares	Theoretical No. of squares
0	79	84.8
1	118	107.0
2	60	67.8
3	33	28.6
4	8	9.0
5	2	2.3
6	0	0.5
	300	300.0

TABLE II

Mitosis Counts

Specimen No.....	1	2	3	4	5	6
Time after operation, *days*.....	3	3	3	2	1	1
	4	1	4	20	41	26
	7	4	1	11	41	28
	8	2	4	15	45	23
	5	3	7	10	40	29
	8	2	6	12	41	40
	7	4	5	16	39	24
	4	2	4	12		
	4	2	3	13		
	9	4	4			
	6	4	3			
Mean.....	6.2	2.8	4.1	13.6	41.2	30.0

Poisson distribution. The results show that there is a very close approximation to the theoretical distribution in sampled areas of the size chosen; and hence that in this specimen there is no systematic tendency of mitoses to occur in groups.

There is in addition no greater tendency of mitoses to appear near

bile ducts than elsewhere. It was also thought worth while, since it seemed on casual observation that cells in the same phase of mitosis (especially prophases) tended to be somewhat grouped, to investigate this from the same map. Hence the phase of mitosis of the nearest dividing cell to each of the 33 prophases was noted. Four prophases, 22 metaphases, five anaphases, and two telophases were found. But three prophases would have been expected on a random basis.

TABLE III

Percentage of Mitosis in Hepatic Cells

Approximate time after operation			
1 day	2 days	2 days	3 days
per cent	*per cent*	*per cent*	*per cent*
2.0	2.2	0.2	0.28
2.4	1.36	0.2	0.36
0.0	1.75	4.4	1.1
1.2	1.2	0.85	0.9
2.0	0.8	0.11	0.62
1.5	2.0	0.5	0.8
0.9	0.35	1.0	0.5
1.3	0.9	1.2	1.1
4.12	2.4	0.8	0.7
3.4	1.3	0.9	0.6
4.2	0.0	1.2	0.41
1.7	0.0	0.9	0.75
3.0	0.05	0.0	0.1
	1.1	1.6	
	1.15	0.0	
	0.4	0.0	
	1.8	0.7	
Mean....2.13	0.97		0.63

A further series of counts from scattered parts of six specimens of regenerating liver are shown in detail in Table II, as a further indication that the distribution of mitoses is reasonably uniform throughout the organ. Each mitosis count is on 1000 adjacent liver cells.

Table III, which shows the percentage of mitoses in 60 consecutive livers taken at periods between 24 and 75 hours after operation, indicates that there is a great variation in the frequency of mitosis in a series of regenerating livers which should be growing at approximately the same rate.

These livers were removed within 3 hours of the 1, 2, and 3 day intervals following operation, and the nearest integral number of days in each case is shown. The mean value for each period is shown, and we note that the individual figures range in each case from zero to two or more times the mean value. The significance of these values will be discussed later.

In view of this extreme variability in mitosis counts done at random

TABLE IV

Percentage of Hepatic Cells in Mitosis

Time	Animal No.									
	1	2	3	4	5	6	7	8	9	10
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
19					0					
20						0				
21					0	0				
22		0								
23			0							
24		0	0	8.15(1)	0.10	0.15				
25	1.23(2)			3.40						
26	3.67(3)									
27				0.83					2.14(4)	
28				0.69						1.29
29									2.08(5)	
30										1.38
32										0.56
38									0.54	2.08(6)
46								1.36		
48							0.93		0.91	
49										2.72(7)
50							0.74	0.72		
54							1.09	0.88		

Figures in parentheses refer to specimens in Table V.

on regenerating livers, it seemed desirable to establish if possible that the mitosis count in a single liver, from which biopsy specimens were taken from time to time, varied similarly.

Table IV shows that this is definitely the case, and also that mitosis can be considered as beginning rapidly after the 24th hour post-operatively.

Since in some of the experiments the mitotic count rises with time,

while in others it falls, there is evidently no constant effect of the operative procedure which could be considered as an important source of error. It is reasonably certain also, from Tables I and II, that the variations are not due to unequal distribution of mitoses throughout the entire liver.

In Table V is shown the distribution of mitoses with respect to phase, in seven of these specimens. It appears that the percentage of mitoses in each phase shows slight changes from time to time, probably dependent on the fluctuations from hour to hour in the total mitoses. There is nothing in these specimens or in any others which we have examined to suggest that a large number of mitoses begin simultaneously throughout the liver.

TABLE V

Percentage of Total Mitosis in Each Phase

Specimen No.	1	2	3	4	5	6	7
Time after operation, *hrs.*	24	25	26	27	29	38	49
Prophases, *per cent*	20.5	15.0	18.7	8.9	15.0	11.6	4.1
Metaphases, *per cent*	48.2	51.2	48.3	55.6	52.5	65.3	51.0
Anaphases, *per cent*	22.2	18.8	20.9	24.4	22.5	15.4	30.6
Telophases, *per cent*	9.1	15.0	12.1	11.1	10.0	7.7	14.3
Total mitoses counted	176	80	91	45	40	26	49

DISCUSSION

Having the raw data above, it is now interesting to consider what is the correlation between mitosis rate and the growth rate of the liver cells as a whole. Since, according to previous work (2), it has been shown that during the interval from 24 to 72 hours after operation the number of hepatic cells in the liver increases from 33.5 to 65.5 per cent of the number of cells originally present, we may assume that for every 1000 cells present at the beginning of this period, 955 mitoses occur during the next 2 days. Since, however, each mitosis count represents the number of mitoses present at a certain time proportional to the number of cells present at that moment (not to the number present at the beginning of any finite period), it is clear that we must have recourse to the calculus in order to correlate these counts with the

growth rate. We must first find a simple empirical expression for the cell growth curve.

In the first part of Table VI is shown the number of cells present in the liver at various postoperative intervals, as per cent of the cells originally present, from data in another paper (2). We are evidently dealing with a rate which is maximal during the 1st day of cell restoration (24 to 48 hours after operation) and decreases constantly thereafter. Moreover, experimental work of various kinds suggests that restoration is correlated with the portal blood passing through the remaining liver parenchyma after operation (6–8).

Whether or not this explains cell multiplication as well as tissue hypertrophy, we may consider as a first approximation for purposes of a mathematical expression, that the rate of cell increase begins maximally and then is constantly retarded so that it remains proportional to the number of cells yet to be formed, approaching the original

TABLE VI

Number of Cells in Liver

Time postoperative, *days*	1	2	3	4	6
Actual No., *per cent*	33.5	52.0	65.5	72.0	81.0
Calculated No., *per cent*	33.4	52.1	65.6	74.8	87.8

cell number as a limit. This type of growth is commonly seen by the embryologist, and would have the general formula:

$$N = C(1 - e^{-kt}) \tag{1}$$

where N represents the number of added cells, t is time, and C and k are constants. According to this formula, when $t = 0$, $N = 0$, and when $t = \infty$, $N = C$, while between these values the increase in N is proportional to an exponentially depreciating quantity representing the number of cells not yet formed. Since N at the beginning is finite, t must be finite also, and we must neglect that part of the curve before the beginning of cell restoration. Since C is the limiting value of N, we may put it equal to the number of cells originally present and express N and C in such terms that $C = 1$. If now we take the first few figures in Table VI, we find that they are satisfied by formula (1), expressing t in days after operation. This can be done by putting

$k = 0.33$ and adding 0.23 to t, in order to set the initial rate at the observed value. This gives the theoretical values in the table.

Thus the empirical formula fits the data through the 3rd day. Beyond this point the growth appears to be retarded more than would be expected from our simple assumption. This might be due to the development of retarding substances or to the fact that the rate may not be directly proportional to the excess blood flow, or to other factors. No reasonable revision of the formula, however, would make any important difference in the growth curve through the 3rd day.

Olivo and Slavich (9) have studied the mitotic incidence in the chick embryo heart, and on correlating this with the growth rate show that the number of mitoses seen is the number which should occur during a 38 minute period; they conclude that this is the average duration of cell division. We have made further calculations for the purpose of

TABLE VII

Interval, *days*	1	2	3
M/A, per cent per day	63.9	30.5	17.1
Mean percentage mitosis	2.13	0.97	0.63
A, days	0.0333	0.0318	0.0369
Duration of mitosis, *min.*	48.0	45.9	53.1

establishing a similar period in our material. Differentiating formula (1), and inserting constants, we obtain:

$$\frac{dN}{dt} = 0.33e^{-0.33(t+0.23)} \tag{2}$$

Now, if we let M represent the proportion of cells in mitosis at any one time and A represent the average duration of a mitosis, we may consider that M/A represents the instantaneous rate of increase in the cell number divided by the number of cells in the liver at that moment. Therefore,

$$\frac{M}{A} = \frac{dN/dt}{N}, \quad \text{or} \quad M = \frac{A \times 0.33e^{-0.33(t+0.23)}}{1 - e^{-0.33(t+0.23)}} \tag{3}$$

According to this, the values of M in terms of A have been estimated for the 1, 2, and 3 day intervals after operation, and are shown in

Table VII, together with the values found in Table III, and the resulting calculated mitosis time.

The most probable mean duration of our mitoses would, therefore, be 49 minutes. This time is a little longer than that which Olivo and Slavich established for the chick embryo heart. Among the sources of error which must be considered are these: (*a*) the personal equation in the matter of judging the earliest prophases and the latest telophases; (*b*) the fact that many mitoses cover more space than resting nuclei and so are likely to be seen in a thin section with greater proportional frequency; (*c*) the fact that the 1 day counts in our material were mostly done at the beginning of mitosis, when in certain cases there seems to be a very high initial count which falls off rapidly (see No. 4 in Table IV). The last two of these possible errors would make the true counts lower than the estimates and so increase the mitosis time. It is worth noting that Olivo and Slavich made their estimates on a basis of 2 day intervals, and that the several figures for these intervals range from 33 to 68 minutes. Moreover, any increase in average cell size before the 20th day would, if allowed for, raise the mitosis time above their estimates.

Various observers have timed the visible duration of mitosis in tissue cultures and have obtained figures of the same order of magnitude as our own. Lewis and Lewis (10) obtained figures of about 30 minutes to 2 hours for fibroblasts, and in the Walker rat sarcoma 338 they found the duration to be about an hour (11). Strangeways (12), with embryo fibroblasts, found the duration to be 34 minutes.

It would be vain to attempt to state the intermitotic period in our cells, since we have no way of knowing whether cell increase is by several divisions of a few cells, or by a few divisions of all cells.

The extreme variability in mitosis rate at various times seems well shown in our data. It appears from figures given by Olivo and Slavich (13) that the same is true in less degree in tissue cultures. As far as can be seen in Table IV, there is little uniformity with respect to time in the various animals of our series, except for the consistent absence of mitosis during the 1st day. There is no reason to suspect that there is an instantaneous mitogenetic stimulus at any time, since the mitotic figures seem in all cases well distributed throughout the several phases; nor is there any evidence of local mitogenetic effects or of local varia-

tions due to differences in the blood supply of the central and peripheral parts of the lobule. Moreover, the random scattering of mitoses does not support the idea that the new tissue is to any great extent derived from bile duct cells.

It is very interesting that no mitosis occurs for about a day after operation. It can be noted that mitosis does not take place until the cells have, on the average, increased to about one and one-half times their usual volume; but it is likewise true that in fasted animals the cell increase continues at nearly the usual rate while the size of the liver is actually decreasing (2). It can be seen that the hepatic cells fill with lipoids at about the time when division begins. The only noticeable morphological change in the cells before the onset of mitosis is that the nuclei become increasingly variable in size and chromatin content. This visible premitotic stage, however, occurs relatively late in the 1st day. The latent period in our material is analogous to the period before growth in fresh explants, which has been found by various observers to increase with the age of the explanted tissue (14). Liver explants, however, will not proliferate at all if taken from animals beyond the latter half of embryo life (15). It has generally been thought that the latent period in explants is the time required for the cells to become dedifferentiated, but the hypertrophying liver in our experiments shows none of the characteristics ordinarily attributed to undifferentiated tissue.

The mitosis rate near the beginning of activity in the liver (21.3 per 1000 cells) is strikingly similar to that in early embryo growth (22.5 in the 2 day chick heart (9)) and to that in well growing cultures of mesenchyme (between 2 and 3 per cent (16)). One might think that this represents the maximal rate at which certain tissues can undergo prolonged cell growth, although we have seen that the instantaneous rate in individual cases can be much higher.

SUMMARY

1. Following partial hepatectomy in the rat, there is a latent period of 1 day during which the rapidly growing organ shows no increase in cell number. Mitosis then begins rapidly, following a brief premitotic period of visible nuclear changes.

2. It can be shown that the increase in cell number during the ensuing 48 hours follows a formula of the type $dN/dt = ke^{-kt}$; beyond this time it is retarded more than this simplified formula would predict. The average mitosis rate at 1, 2, and 3 day intervals after operation follows the same formula; from this the duration of each mitosis is calculated to be about 49 minutes. It is not necessary to assume that amitotic division plays an important part, and no such divisions have been seen by the writers.

3. The percentage of cells in mitosis in a single hypertrophying liver varies widely from hour to hour, so that a single mitosis count tells nothing about the growth rate. The fluctuations occur at different times in different livers. It appears that no great number of mitoses begin or end simultaneously.

4. Mitoses are evenly distributed throughout the liver and throughout each lobule; there is no preponderance near the bile duct cells.

5. The mean initial mitosis rate (at 24 hours after operation) is 2.13 per cent, and it diminishes from then on. This rate is very similar to that in early embryo heart and tissue cultures of mesenchyme. In individual specimens the rate can be over 8 per cent. This rapid rate occurs without signs of cell dedifferentiation.

BIBLIOGRAPHY

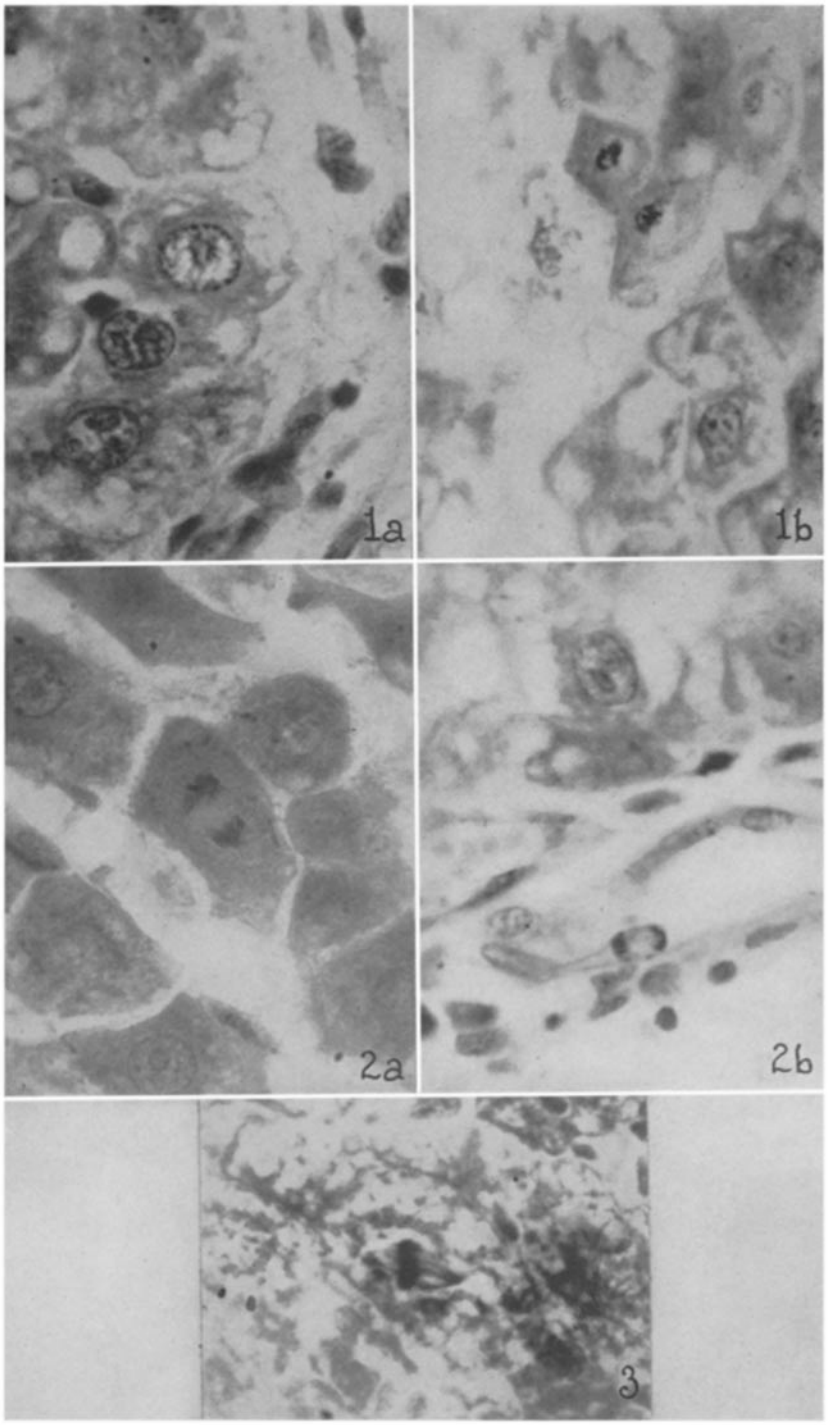
1. Higgins, G. M., and Anderson, R. M., *Arch. Path.*, 1931, **12,** 186.
2. Brues, A. M., Drury, D. R., and Brues, M. C., *Arch. Path.*, 1936, **22,** 658.
3. Thuringer, J. M., *Anat. Rec.*, 1928, **40,** 1.
4. Warren, S., *Am. J. Path.*, 1933, **9,** 781.
5. Ludford, R. J., *Proc. Roy. Soc. London, Series B*, 1928, **102,** 397.
6. Higgins, G. M., and Anderson, R. M., *Arch. Path.*, 1932, **14,** 482.
7. Stephenson, G. W., *Arch. Path.*, 1932, **14,** 484.
8. Higgins, G. M., Mann, F. C., and Priestly, J. T., *Arch. Path.*, 1932, **14,** 491.
9. Olivo, O. M., and Slavich, E., *Arch. Entwcklngsmechn. Organ.*, 1929, **121,** 96.
10. Lewis, W. H., and Lewis, M. R., *Anat. Rec.*, 1917, **13,** 359.
11. Lewis, W. H., and Lewis, M. R., *Am. J. Cancer*, 1932, **16,** 1153.
12. Strangeways, T. S. P., *Proc. Roy. Soc. London, Series B*, 1922, **94,** 136.
13. Olivo, O. M., and Slavich, E., *Arch. Entwcklngsmechn. Organ.*, 1929, **121,** 408.
14. Needham, J., Chemical embryology, New York, The Macmillan Co., 1931, 463.
15. Lewis, W. H., and Lewis, M. R., in Cowdry, E. V., General cytology, Chicago, University of Chicago Press, 1924, 391.
16. Olivo, O. M., *Arch. exp. Zellforsch.*, 1931, **11,** 273.

EXPLANATION OF PLATE 1

FIG. 1. Early prophase (*a*) and late telophase (*b*). These figures represent the approximate limits between which cells have been counted as in mitosis. Iron hematoxylin-eosin. ×900.

FIG. 2. Anaphases in hepatic cell (*a*) and in bile duct cell (*b*). Iron hematoxylin-eosin. ×900.

FIG. 3. Spindle in hepatic cell mitosis. Phosphotungstic acid hematoxylin. ×900



(Brues and Marble: Mitosis in liver restoration)