MITOCHONDRIAL CHANGES IN THE LIVER
OF ESSENTIAL FATTY ACID–DEFICIENT MICE

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ABSTRACT

Livers of mice on diets deficient in essential fatty acids (EFA) have been studied by light and electron microscopy. The most conspicuous changes occur in the mitochondria. In light microscopy the mitochondria appear very much enlarged in the periportal region of the lobule. In electron micrographs they have additional cristae, sometimes very abundant, ranged in stacks in the central cavity. The matrix may be more electron-opaque than normal. This is in contrast with the enlarged mitochondria appearing under other experimental conditions, where the cristae are reduced in number and the matrix is less electron-opaque. It is known that there is an uncoupling of oxidative phosphorylation in EFA-deficient mitochondria. As a hypothesis it is proposed that the uncoupling may be due to a molecular defect caused by the absence of EFA in the structure that determines the spatial relationship between the electron transport chain and oxidative phosphorylation. It is further tentatively suggested that the changes in mitochondria may be attributed to lack of ATP. The possibility is discussed that the mitochondrial changes are ineffective attempts at compensation for this lack.

INTRODUCTION

There is abundant evidence in the literature that the mitochondria of animals deficient in the essential fatty acids (EFA) are abnormal. One proposed role of the essential fatty acids is to contribute to the structure of phospholipids, particularly those that become constituents of the membranous structures of the cell, including the mitochondria. There are two recent extensive reviews on essential fatty acids (1, 26), both of which discuss their possible significance in cell structure. Isolated mitochondria from the livers of EFA-deficient rats have an increased tendency to swell over those from normal livers, are more fragile (18), and have a lower P:O ratio, which indicates an uncoupling of oxidative phosphorylation (15). The swollen, isolated mitochondria appear as spheres and crescents. There has been one study (18) of mitochondria of EFA-deficient livers in fixed preparations. In spite of some difficulty in interpreting the preparations, two impressions were reported: first, that the mitochondria of fat-deficient livers were slightly larger than normal; and second, that the unusually large round forms and crescents seen in isolated mitochondria do not occur in the intact cell.

Our investigation was undertaken because of the repeated statement that essential fatty acids are necessary for the production of new cells and growth of tissue (12, 26). The incidental observation of very much enlarged mitochondria in the livers of some EFA-deficient mice led us to a more extensive study, with both the light microscope and the electron microscope, of the mitochondria in EFA deficiency.

MATERIAL AND METHODS

The mice used were of the inbred BUB strain raised in this laboratory and maintained at 78°F. Breeding
FIGURES 1 AND 2
Light microscope photomicrographs of Regaud preparations for revealing mitochondria. X 1125.

FIGURE 1
Control liver of a mouse maintained 85 days on experimental diet supplemented with essential fatty acids. Normal size and shape of mitochondria in cells around portal vein.

FIGURE 2
Periportal cells of liver of an EFA-deficient mouse, 53 days on the experimental diet. Mitochondria vary in size from cell to cell and even intracellularly, some attaining marked enlargement.

FIGURES 3 AND 4
Low magnification electron micrographs of periportal cells of the same livers as those in Figs. 1 and 2, for comparison of mitochondrial size. X 6000.

FIGURE 3
Control, EFA-supplemented diet.

FIGURE 4
Enlarged mitochondria, EFA-deficient mouse. Two of the mitochondria in this cell enlarged further in Fig. 9.
mice were fed Purina laboratory chow, whereas pregnant and nursing females received Purina breeding chow.

Our studies of essential fatty acid deficiency in the mouse have been carried out over a period of 5 years. During this time we have employed 25 groups of weanling mice, 18 to 24 days old, 12 to 22 animals in each group, with initial weights varying from 8 to 10 gm, 10 to 12 gm, and 10 to 15 gm. The initial weight of the mouse is of great importance. The smaller the animal, the greater the requirement of EFA, and consequently the deficiency symptoms appear earlier and more uniformly throughout the group. Animals of both sexes were used. Whereas in the rat the requirement of EFA is higher in the male than in the female, in the mouse the females are as susceptible as the males to EFA deficiency, if not more so.

Several EFA-deficient diets were employed. Most of them, based on that of Decker et al. (5), had the following composition: vitamin-test casein, 20 per cent, with added vitamins; sucrose, 72 per cent; salt mixture, 4 per cent; non-nutritive fiber, 4 per cent. Different carbohydrates were tried in the place of sucrose. Best results, with respect to rapidity of appearance of deficiency symptoms, were obtained with sucrose, with dextrose, and with a 2:1 mixture of potato starch and sucrose, in that order.

With EFA-deficient diets the total body weight of the growing mouse reaches a plateau below normal adult weight. This has proved to be the most reliable indication of EFA deficiency. Skin symptoms sometimes, but not always, appear much later and are more variable even among siblings. These external modifications include thinning and eventual loss of fur around the eyes, mouth, chin, and abdomen, and failure of sores to heal once they occur. We attribute these skin lesions to local abrasions which do not heal normally; their great variability is thus due to the variability of injuries obtained from food cups, fighting, etc. Therefore, no attempt has been made to equate skin symptoms with cytological changes in the liver.

When 8- to 9-gm mice are fed an EFA-deficient diet containing sucrose, their weight begins to plateau after as early as 10 to 15 days on the diet; with dextrose, this occurs after 20 to 25 days. Growth continues very slowly, if at all, thereafter: males reach a final weight of 22 to 26 gm, whereas their male siblings receiving corn oil supplements reach a total weight of 34 to 36 grams; females reach the same 22- to 26-gm range, whereas control females attain 30 to 32 gm, after 50 days on the corn oil-supplemented diet.

For this electron microscope study of the liver mitochondria three series of mice were employed as follows: The group of mice on a sucrose-containing diet were sacrificed after 15, 30, 45, 58, 75, 79, and 87 days; those on a dextrose-containing diet, after 14, 21, 28, 35, 42, 51, 53, 57, 85, and 92 days; those on a sucrose plus potato starch diet, after 21, 32, 39, 49, 64, 71, 80, and 101 days. In addition, the livers of 17 other EFA-deficient and control mice were taken from several other experimental groups after the animals had been on the diet for 50 to 80 days when mitochondrial abnormalities in the liver are most pronounced.

The livers of these animals were prepared for optical microscopy by Regaud's method for mitochondria, fixed in 10 per cent buffered neutral formalin, and stained with Sudan black B for lipids. For electron microscopy they were fixed for 1 hour in 2 per cent osmium tetroxide in Millonig's phosphate buffer, dehydrated in alcohol, and embedded in an 85:15 mixture of butyl and methyl methacrylate containing 1 per cent benzoyl peroxide, or in Epon. Sections were cut on a Porter-Blum microtome with glass knives (2), stained with Karnovsky's lead method A (14), and examined with a Siemens Elmiskop I electron microscope.

**OBSERVATIONS**

The initial observation with the light microscope revealed that in some of the mice on EFA-deficient diets the mitochondria of the liver cells may be much enlarged (Figs. 1 and 2). This occurs first in the peripheral zone of the lobule and may extend for varying distances toward the central vein. This enlargement is apparently more con-
spicuous than that reported by Levin et al. (18). It is to be emphasized that the mitochondria vary in size considerably from cell to cell in the same liver (Fig. 2). Furthermore, in the ultrastructural alterations described below, we cannot present a precise sequence of changes with respect to time because of individual differences, even among siblings on the same diet, and very marked differences in rate of change induced by various diets which vary only slightly in composition (27).

The most striking cytological change revealed by the electron microscope is an increase in the number of cristae in the enlarged mitochondria. The marked mitochondrial enlargement which may occur in periportal cells is illustrated by a comparison of the mitochondria in normal liver (Figs. 3 and 5) and in EFA-deficient liver (Figs. 4 and 6), prepared in the same way and photographed at the same magnification. It is not unusual to find a single mitochondrion occupying the entire width of the cytoplasm between the nucleus and the cell membrane or bile canaliculus (Figs. 6 and 9). We must emphasize, however, that the degree of enlargement may vary from cell to cell, as was seen with the light microscope (Fig. 2), and even in a single cell (Figs. 7 and 9). The matrix of the enlarged EFA-deficient mitochondria is as dense as, and often more dense than, that of controls.

The cristae in normal liver mitochondria, as described by other investigators (7, 21, 24), are not abundant (Fig. 5); they are clearly continuous with the internal mitochondrial membrane (Fig. 5, large arrows) and, even in only slightly vesiculated mitochondria, they do not extend all the way across the internal matrix (Fig. 3). In EFA-deficient livers the usual short cristae around the periphery of the organelle are present and in the very much enlarged mitochondria they may be even shorter than normal, as described in other types of enlarged mitochondria (24). In addition, there are new cristae, unusually long, oriented in stacks of parallel lamellae within the center of the matrix (Figs. 6, 7, and 9). These central cristae have no obvious attachments to the inner membrane. However, the frequent presence of small, circular or oval profiles of cristae in the matrix around the central stacks of elongated cristae and the occasional presence of stacks near the mitochondrial membrane suggest that such attachments do exist (Fig. 9). The additional cristae sometimes nearly fill the center of the mitochondrion (Figs. 6 and 7), but often they do not (Fig. 9). These cristae occur in some small mitochondria (Fig. 7, top). They would seem, therefore, to appear before mitochondrial enlargement. Furthermore, although all enlarged EFA-deficient mitochondria contain the additional central cristae, mitochondria which are enlarged to approximately the same extent after several days of fasting (4, 7, 9) do not contain them (compare Figs. 8 and 9). Hence, the presence of the central cristae is independent of mitochondrial enlargement.

Other membranous structures, i.e., the endoplasmic reticulum and associated nuclear envelope, Golgi apparatus, and cell membrane, seem unchanged. An increased number of microbodies (Figs. 12 and 13) may be evident during the early stages of EFA deficiency when central cristae are first detected and before mitochondrial enlargement is evident, but few pericanalicular dense bodies (lysosomes?) are present. There is a marked accumulation of sudanophilic lipid which in light microscopy appears at first most concentrated in the periportal zone of the lobules and later extends throughout the lobule. After methacrylate embedding the lipids in control livers of mice that received EFA supplements in their diets are homogeneous and dense (Fig. 10), whereas the lipid droplets in EFA-deficient livers appear abnormal in that they are no longer electron-opaque except for a ragged network condensed at the surface of the droplet (Fig. 11). These differences are not evident in Epon-embedded tissues. Lipid changes are being reserved for further study, however. The changes in mitochondrial membranes appear the same in methacrylate- and Epon-embedded livers.

**Figure 7**
Liver of an EFA-deficient mouse. One small mitochondrion (top of page) and two moderately enlarged ones (upper center and bottom right) contain additional, central cristae, whereas others appear relatively normal. Note, however, circular and oval cross-sections of cristae in some of smaller profiles of mitochondria (arrows). X 35,000.
Mitochondrial changes always begin in the portal zone of the lobule and gradually extend toward the central vein. As noted above, in the early stages of EFA deficiency the swollen mitochondria or small ones with additional cristae mingle with normal ones in the same cell (Fig. 7), but at later stages the portal cells contain abnormal mitochondria almost exclusively (Fig. 4). In the latter case the total number of mitochondria per section is certainly reduced, but our material does not lend itself to precise quantitative study. The cytological changes are not evident up to 30 days on the diets; thus they occur after the body weight reaches a plateau. In some mice, but not in all, they begin to appear between the 35th and 45th day. However, the changes are usually present in all mice between the 50th and 60th day; they may be very pronounced in the portal zone and more extensively distributed in the lobule. During this period the mice often die. In the mice that survive up to 90 to 120 days, more cells show changes and more mitochondria per cell are involved but precise quantification of the number of cells containing abnormal mitochondria has not been attempted.

Because of the nature of the deficiency and the many factors to be controlled in its production, it has been difficult, in general, to obtain statistically valid, uniform results. The mouse at weaning may weigh as much as \( \frac{1}{3} \) to \( \frac{1}{2} \) of its adult weight. Its essential fatty acids are so tenaciously conserved that its requirement of additional EFA is small. Traces of EFA in the diet, removed only with difficulty, or in the feces and recovered by coprophagy, or possibly even synthesized at a very slow rate in the body, all introduce variations almost impossible to control. Furthermore, the initial supply of EFA must vary unpredictably in individual mice. Even the observable skin symptoms vary with environmental changes, especially changes in humidity. These problems are discussed elsewhere (27). The changes we have observed in the mitochondria of the liver have occurred regularly in animals that display the characteristic symptoms. We have not seen such changes in liver mitochondria under other experimental conditions nor have we found any reports of such changes in the literature. We have, therefore, felt justified in attributing them to this deficiency.

**DISCUSSION**

The observations reported here were unexpected. That the deficiency involves fatty acids important in the structure of the membranes has been stated by several investigators, but the only organelle affected, as far as could be discerned by light and electron microscopy, is the mitochondrion. All the other membranous structures appear normal. Contrary to what might be expected, rather than appearing defective, the mitochondria are enlarged and the cristae are very much elaborated.

The most important physiological effect of the EFA deficiency is the uncoupling of oxidative phosphorylation. Other symptoms seem to be directly or indirectly a result of this effect. The uncoupling should itself be attributed to some defect of the mitochondria, especially of the mitochondrial membranes, since the entire mechanism of intracellular oxidation and oxidative phosphorylation is known to be located in these membranes. The basic problem would then seem to be one of determining why a membrane defective in molecular structure and in function should be normal in appearance and the structures formed more extensive and elaborate than normal.

The mitochondrion is considered to be made up of many units, each with a chain of oxidation-reduction components, coupled to oxidative phosphorylation (17). The efficiency of each unit must be determined by the stability of the arrangement of its components. That lipids are involved in the structural integrity of the mitochondrion and its units has been repeatedly suggested (6, 10, 23). Lipids are considered to enter importantly into the structure of the ETP of Green (11) and possibly play a role in the geometry of the respiratory assemblies of Lehninger (16). Green has further suggested that the molecules of the functional compo-
nents may be associated with specific lipids. It has been proposed that EFA are specifically involved in the coupling of oxidative phosphorylation to the electron transport chain (19). The observation that the coupling structure is first attacked when isolated mitochondria are exposed to snake-venom phospholipase (22) indicates its sensitivity. It is to be emphasized, however, that the subject under discussion here is not the mechanism of oxidative phosphorylation, but simply the structural framework in which it is carried out.

The evidence that EFA play an important role in the composition of the phospholipids that enter into the structure of the cell, especially of its membranes, has been summarized by Macmillan and Sinclair (19). In EFA deficiency, other polyunsaturated fatty acids, for example, a trienoic acid of the oleic acid series, take their place (20). In most membranous structures they seem to be adequate substitutes. That they are not adequate in the mitochondrial membranes, however, may be explained on the basis of the special role the lipids and lipoproteins play in these membranes. It is generally agreed that lipids are specifically involved in electron transfer in the energy-producing mechanisms. Since uncoupling occurs regularly in EFA deficiency, it may be concluded that fatty acids of this specific molecular configuration are important in maintaining the spatial relationship between the main electron transport chain and oxidative phosphorylation. The deficiency involves, then, not a gross defect in the structure of the membranes, in which other unsaturated fatty acids seem adequate, but a defect in the molecular configuration of the fatty acids on which normal function depends. That there is no such specific function of unsaturated fatty acids in other cellular structures would explain the fact that they are not visibly or functionally altered in EFA deficiency.

In the development of EFA deficiency, the replacement of the EFA by other unsaturated fatty acids in the mitochondria must take place gradually. Hence, the uncoupling of oxidative phosphorylation must be gradual and progressive, not only in the cell but also in a given mitochondrion. Correspondingly, the production of ATP must fall off gradually. If the visible changes in the mitochondria depend on deficiency of ATP, they would take place only when its concentration has attained a critical minimum value. This would not necessarily occur in all mitochondria in a cell at the same time or at the same rate, hence the great variation in size of mitochondria in some cells (Fig. 7).

Any attempt to explain the mitochondrial changes, in light of the considerable knowledge of their function and the highly sophisticated theories of the relationship of their structure to function (6, 10), must be, to a considerable extent, speculative. The events taking place in suspensions of isolated EFA-deficient mitochondria are similar to those occurring in other suspensions sooner or later, but they happen so quickly that it seems obvious that they must be the result of changes within the intact cell. Since enlargement of mitochondria and uncoupling occur together, there has been some debate as to which is cause and which is effect. It seems probable that, in suspensions of normal mitochondria, the first change is decreased ATP production due to lack of substrate (16). Water taken into the mitochondrion by osmosis is normally removed by the action of ATP, possibly through an actomyosin-like contraction serving as a pump. In the absence of ATP, the water accumulates and causes swelling of the mitochondrion. This leads to structural changes in the membranes, possibly only stretching, followed by uncoupling. The two processes, therefore, swelling and uncoupling, would then go on together, each expediting the other. In the mitochondria of EFA deficiency the uncoupling would already have occurred, or at least begun, before the mitochondria are isolated. Hence, with the deficiency of ATP, they begin to swell immediately.

Enlargement of mitochondria in intact liver cells has been reported in several experimental conditions, for example, in fasting (4, 7, 9) and ischemia (13). This enlargement is different from

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**Figure 9**

Enlarged, cristae-enriched mitochondria of the liver of an EFA-deficient mouse shown in Fig. 4, for comparison with liver mitochondria of fasted mouse in Fig. 8. There are several stacks of central cristae with different orientations. Between them and the inner membrane are numerous circular profiles of cristae. × 35,000.
the swelling observed as a decrease in optical density in suspensions of isolated mitochondria, although it may be a related phenomenon. The enlargement in vivo in EFA deficiency seems to be unique in that the cristae are greatly increased in number, whereas in other conditions the cristae may be reduced and shortened. The difference is illustrated in Figs. 8 and 9 where greatly enlarged mitochondria in the livers of fasted and EFA-deficient mice are contrasted. Since there is also an increased number of centrally situated cristae in normal sized as well as in enlarged mitochondria in EFA deficiency (Fig. 7), the mitochondrial enlargement is probably an independent phenomenon. In some conditions, for example, in ischemia (13), mitochondrial enlargement seems to be the result of disturbance of water-electrolyte equilibrium between mitochondria and the cytoplasm, probably due to lack of ATP. The actual enlargement in EFA deficiency might be related to this. However, in ischemia (13) the matrix of the mitochondria is much less dense than in normal mitochondria, which suggests dilution, whereas in EFA deficiency it is usually somewhat denser than normal, which would imply accumulation of new material.

As an alternative to osmotic swelling, we had considered the possibility that enlargement may be due to fusion of small mitochondria. This has been repeatedly reported in living cells in tissue culture (reviewed by Novikoff (21)). The additional cristae are found to be in groups parallel to each other in the center of the matrix. In some mitochondria there may be several groups of cristae (Fig. 9), each of which, if fusion had occurred, might have come from a different mitochondrion. There are, however, many small circular profiles between the central clusters and the inner membrane (Fig. 9). We are not sure whether they represent small tubular cristae, as suggested by Fawcett for occasional similar profiles in normal liver cells (Fig. 5, small arrows), or thin, tubular necks connecting the plate-like cristae, that usually appear to be free in the matrix, to the inner membrane. If they represent the latter, it is possible that these cristae are simply crowded out of the surface of the inner membrane and that the grouping is an expression of some physical property.

A final suggestion, perhaps the most speculative, is that the enlarged mitochondria with additional cristae represent true growth, an increase in the mitochondrial material of the cell. It is possible that they represent a structural adaptation, an attempt on the part of the mitochondrion to produce functioning components to replace those that have become useless in the deficiency. A similar concept has been developed by Chêvremondt and Chêvremondt-Comhaire (3) and elaborated by Frederic (8). Their suggestion is that there is an equilibrium between the total amount of mitochondrial substance and amount of cytoplasm as a whole. In our case it is the number of cristae rather than the number of mitochondria that is increased. It is generally stated that the number of cristae of mitochondria corresponds with cellular activity (21). There would be need, then, of more cristae if some of them were for any reason functioning improperly. The question of the mechanism of this increase would possibly be related to the larger one of the origin of new mitochondria and the factors involved in producing and maintaining the proper number in cells. Some mechanism must exist by which the "normal" number is maintained. This may very well depend upon a negative feedback, the key to which is the availability of ATP in the cell as a whole.

**Figure 10**
Liver of control mouse fed 60 days with an experimental diet supplemented with EFA-rich corn oil. Lipid droplet (L) is uniformly homogeneous and electron-opaque. × 35,000.

**Figure 11**
Liver of an EFA-deficient mouse whose liver was laden with sudanophilic lipid droplets. The lipid (L) is not electron-opaque, as though some component had dissolved out leaving only an irregular dense network at the periphery of the nodule. Note tangential section of the edge of one of the lipid droplets (arrow). A relatively large microbody (mi) is present. × 35,000.
ATP is necessary elsewhere in the cell in order to supply energy for, among other activities, transport of materials through the cell and to prevent intracellular edema by pumping water out of the cell. Somehow the production of ATP must be geared to the demands of the cell. If this is so, a sustained drop in ATP production might "stimulate" the development of new cristae in all mitochondria, not only in those in which uncoupling is great enough to result in swelling. If, in the absence of EFA, the new cristae are ineffective as far as ATP production is concerned, the demand would result in the production of more and more cristae, resulting in the greatly increased number of cristae in the EFA-deficient mitochondria.

There should also be an attempt to produce new mitochondria. Microbodies which contain laminae that may be strikingly reminiscent of clusters of cristae are, in a few cases, abundant. This led us to consider that they possibly represent a step in the production of new mitochondria, as Rouiller and Bernhard (25) proposed. If this is so, however, mitochondria are not produced constantly or abundantly in this way throughout the development of the deficiency, for in the majority of cells the number of microbodies is not unduly increased. On the other hand, their occasional great abundance, particularly during early phases of the deficiency before mitochondria are very much enlarged (Figs. 12 and 13), may indicate a discontinuous process of mitochondrial formation from microbodies.

The relatively dense matrix of EFA-deficient mitochondria suggests another possibility. Fawcett (7) has proposed that new cristae may be developed from matrix material. If so, in the normal reproduction of mitochondria the matrix may contain material for the production of new cristae in response to the need for ATP in the cell as a whole, and when the number of cristae reaches a certain size the mitochondrion may divide. Such "dividing" mitochondria have repeatedly been described (reviewed by Novikoff (21)). If the "division," like other activities of the mitochondrion, requires energy, however, the deficiency of ATP might account for a failure to divide and, thus, for both the increase in size and the unusual accumulation of cristae.

This concept, admittedly speculative, may be summarized as follows: the important consequence of continual deficiency of EFA in the diet is the failure to maintain the normal fatty acid composition of the lipids in the membranes of mitochondria. In spite of the fact that these fatty acids are tenaciously conserved, they must gradually be lost, and, as in other membranes, replaced by unsaturated fatty acids that can be synthesized in the body. This produces an important molecular defect in the mitochondria because these new fatty acids cannot participate in the specific role that EFA play in the function of the membranes. The defect leads to the uncoupling of oxidative phosphorylation and consequent failure to produce ATP. Without this source of energy, mitochondria immediately swell when isolated. This swelling may involve mitochondria already enlarged in the living cell as well as normal-sized ones. The criterion of swelling in vitro would not distinguish between them. Enlargement in vivo would in part at least appear to be a different phenomenon. The failure to produce sufficient ATP to provide for the needs of the cell as a whole might well stimulate growth of new mitochondrial material in the form of additional cristae. Since in the absence of EFA the new mitochondria would be defective, the stimulation would be continuous. While the increase in the number of cristae and in the size of mitochondria should lead to division of normal mitochondria, this could not occur in the EFA-deficient ones because of the lack of ATP to furnish energy necessary for division. Speculative as it is, this hypothesis would seem to explain the anomalous situation that enlarged and more elaborate mitochondria result from a deficiency in an important molecule. We offer no suggestion to

**Figures 12 and 13**

Cells of EFA-deficient livers in which large numbers of microbodies (arrows) occur throughout the cytoplasm. These microbodies are structurally similar to those in livers of control mice, that is, they are characterized by a single external membrane, dense matrix, and an internal denser structure which may appear as stacked, straight laminae or as an irregular thread-like form, or both (see that at top of Fig. 13). Mitochondria appear normal in Fig. 13; the mitochondrion and the microbody adjacent to it in Fig. 12 are slightly enlarged. X 35,000.
account for the role of a polyunsaturated fatty acid with a precise configuration in the coupling of oxidative phosphorylation with the electron transport chain.

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27. WILSON, J. W., manuscript in preparation.

This investigation was supported by Research Grant C-510 from the National Cancer Institute, United States Public Health Service.

Received for publication, June 13, 1962.