A BACTERIAL GROWTH INHIBITOR FROM HUMAN CELLS IN CULTURE: TENTATIVE IDENTIFICATION AND POSSIBLE SIGNIFICANCE

BY

BRIAN GROVE SPARKES, B.A., M.Sc.

A thesis submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy at the University of Ottawa, Department of Biology.

Ottawa February 1973

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

There are many who deserve much praise for the stimulation and encouragement to the author at various stages of this work. These include colleagues and fellow students who have made a lasting impression on the author by their curiosity and eagerness to comprehend unconventional ideas. Primarily my deep respect and thanks go to the man who made it possible for this research to begin and who helped it on its way. Dr. C. Paul Kenny deserves the credit for first recognizing that bacteria failed to grow and infect a tissue culture monolayer whose medium had nourished it for a few days. Without his contagious enthousiasm and effort the task of answering "why" would have hardly continued. Equally deserving of my greatest respect and admiration is the man who made it possible for this work to come to a fruitful conclusion, Dr. J. Gordin Kaplan. Nothing great enough on my part can reward his kindness, encouragement, patient understanding and leadership in saving this work and the author from oblivion. Also my sincere thanks go to Drs. Morris Kates and Donn Kushner whose wise criticisms and suggestions helped to develop a worthy frame of reference in the work. My deepest thanks also go out to Dr. Quentin LaHam who deserves much praise for his moral and logistic support.

ABSTRACT

Research was cited showing that dicarbonyls and some derivatives could inhibit tumor growth, viruses, bacteria and animal cells.

The results of this work describe an assay of bacterial growth inhibition by a factor from HeLa cells found in the Culture Conditioned Medium (CCM). Virulent strains of bacteria were more resistant to inhibition by CCM than were less virulent strains. However virulent strains did not destroy the inhibitor more than less virulent strains although they could have been resistant by avoiding entry of the inhibitor. Isolation of the inhibitor by Sephadex indicated it may have a molecular weight of 1000. But inhibitory activity of a smaller component was liberated from larger material. Alcoholic solutions gave electronic spectra resembling those of ketoaldehydes. A ketoaldehyde was isolated as its dinitrophenylhydrazine derivative and was tentatively identified as 4-hydroxy,2-ketobutyraldehyde. Synthesis of this compound implied its identity with the natural product by spectral and bioassay similarities. The compound was a substrate for glyoxalase. It could inhibit the growth of mammalian lymphoblasts. The compound was found to exist in the hemiacetal configuration in non polar solvents and to open in the presence of ions.

The difference between virulent and less virulent bacteria was discussed in relation to the ketoaldehyde. Also the interpretation of electronic and infra red spectra was explained for ketoaldehydes, and the structural implications of the compound were considered.

LIST OF TABLES

FIGURES

PREFACE

The work reported in this thesis begins with a discovery. It is not as if it were a planned set of experiments arising out of a broad body of published scientific data and aspiring to detail what the background implies or gleaning what was left aside.

The idea of each experiment led directly from the preceding one and no whole order was planned beforehand. Keeping the work unified however was the singular goal of identifying chemically that which was discovered to have biological activity. This, of course, the classic role of the Biochemist, is matching structure with function. The Introduction to this match then can only relate the interesting context of the work and is not a rational basis for its initiation. The context discussed here gives the historical significance of the dimension in which the subject finds itself, and tells of similar observations made and pursued, however inadequately, up to the present time. This way the reader gains from the Introduction a perspective capable of enlightening the meaning of what follows; the meaning itself is revealed in the Discussion.

PROLOGUE

" The mental features discoursed of as the analytical are, in themselves, but little susceptible of analysis. We appreciate them only in their effects. We know of them, among other things, that they are always to their possessor, when inordinately possessed, a source of the liveliest enjoyment. As the strong man exults in his physical ability, delighting in such exercises as call his muscles into action, so glories the analyst in that moral activity which disentangles. He derives pleasure from even the most trivial occupations bringing his talents into play. He is fond of enigmas, of conundrums, of hieroglyphics; exhibiting in his solutions of each a degree of acumen which appears to the ordinary apprehension preternatural. His results, brought about by the very soul of method, have, in truth, the whole air of intuition. The faculty of resolution is possibly much invigorated by mathematical study, and especially by that highest branch of it which, unjustly, and merely on account of its retrograde operations, has been called, as if par excellence, analysis. Yet to calculate is not in itself to analyse.....

The analytical power should not be confounded with simple ingenuity; for while the analyst is necessarily ingenious, the ingenious man is often remarkably incapable of analysis. The constructive or combining power, by which ingenuity is usually manifested,..... has been so frequently seen in those whose intellect bordered otherwise upon idiocy, as to have attracted general observation among writers on morals. Between ingenuity and the analytical ability there exists a difference far greater indeed, than that between the fancy and the imagination, but of

a character very strictly analogous. It will be found, in fact, that the ingenious are always fanciful, and the truly imaginative never otherwise than analytic. "

Edgar Allan Poe

from "The Murders In The Rue Morgue"

INTRODUCTION

INTRODUCTION

FOREWORD.

One of the tenets of homeostasis is that the body can take care of itself. Physicians have depended on this to be quite true in most cases and research into the manifestations of this phenomenon has proven useful. Various defense and restorative mechanisms of the body have been discovered and it is these which physicians attempt to encourage when effecting a cure. To treat infections the physician uses whatever medications are appropriate to help the body beat off the invading viruses or bacteria. Sometimes, although perhaps less often than is claimed, the intervention of the physician is decisive, sometimes it is secondary. But the fact remains that the battle against infection is never less than a collaboration. Unless the forces of the patient's own system are deployed in fighting the disease, all the physician's medicines will be useless. Cancer however, does not, on the whole, improve. This could immediately imply that the body's own defence for cancer is not operating. That small percentage of cases of remission of cancer, termed 'spontaneous', could indicate the re-enlistment of the body's defence mechanism. In spite of the implications of a natural defence, a century of cancer research and decades of radiation and surgical intervention have failed to improve survival statistics significantly (173). According to one of the oldest and certainly simplest definitions cancer is viewed as 'uncontrolled growth'. Even this simple view implies that the 'normal growth' is under the 'control' of certain mechanisms operating in the living organism. Seemingly then when the control mechanism is off cells would tend to proliferate. Cancer research has tended to look into the cancer cell to study division rather than to look into the normal cell to study the

reason for non-growth, or for controlled growth.

In retrospect it appears appropriate that cell multiplication is the main characteristic of a primitive acellular or unicellular organism limited only by the availability of the nutrients in its environment. As the organisms evolved, and cells aggregated to form multicellular organisms, some control had to evolve too which inhibited the division of each component cell for the benefit of the organism as a whole. Yet this control on cell division had to be capable of being turned off in the event that cells were damaged and new cells were required by division of the surviving immature cells. This particular mechanism has so far eluded researchers.

The discovery of the role of hormones in the growth and function of certain glands and organs indicates that at least some of the growth regulatory mechanisms operate at the molecular level, that is they are chemical in nature. Our knowledge of the molecular basis of growth suggests that whatever the primary causes of cancer may be, whether viruses, carcinogens, traumas, radiations, or hormone imbalances, the abnormal growth results from a biochemical lesion leading to the deficiency of a regulating factor. The corollary to this hypothesis is the assumption that normal mature cells contain all the 'normal' enzymes, metabolites and other factors necessary for regulation of growth. It would seem plausible that such factors that are involved in growth regulation could be extracted from normal animal tissues and could be recognized by inhibitory effects in animal tumor, normal cell, or bacterial assay systems. The following historical record deals with this plausibility. The early work, in the first half of the century, is reported here very briefly in order to put emphasis on the more recent

evidence. References are discussed in more or less chronological order.

I. Growth Inhibitory Activity of Tissue Extracts

A. Anti tumor extracts.

Ever since the early part of this century, numerous reports appeared in the clinical literature claiming the 'therapeutic effects' of various crude animal tissue extracts in the treatment of cancer patients and experimental tumors. This work began with the desire to enlist the body's own defences against cancer. In this case it was the hope that "immunity to cancer" could be built up by injecting tumor tissue homogenates or extracts, into test animals. The test of "Immunity" was the ability of the animal to take or reject a transplantable tumor. The results of Borrel (51) and Hertwig (142) in 1907 showed they were unable to induce any "resistance" in their experimental animals but Ehrlich(98) in 1908 reported successful results. That same year Gierke (125) reported failure but Lewin (191) in 1909 agreed with Ehrlich. Konigsfeld (177) reported in 1914 that he was also successful but Frankel and Furer (115) in 1915 failed to "induce resistance". Kepinow (167) however, did report the "induction of resistance" in 1920 but Wood (345) in 1925 produced no "immunity". The inoculations given in these studies varied considerably, including homogenates and extracts of pulped, autolysed or irradiated tumor tissue.

The great diversity of experimental procedures among these investigators made it, therefore, impossible to compare their results and to draw definite conclusions on the immunizing power of tumor tissues. However a quick survey of results from this period uncovers some important variations with implications for much later work.

Coincidental with the work on tumor inoculations there appeared a body of other workers who were investigating the "immunizing power" of

other tissues besides tumors. This type of approach grew out of the use of controls for inoculating tumors. Many tissues were used as sources of injectable material but of particular interest were normal spleen and liver. Levin (189) in 1909 concluded he induced "resistance" to a subsequently transplanted tumor by injecting into susceptible rats the autolysed liver of rats naturally "resistant" to tumors, and also in 1910 by injecting normal skin and spleen preparations (190). The success of non-tumor tissue here could not possibly support the idea that specific tumor antigens are responsible for "immunity", but the success of spleen tissue does not rule out some immunological reaction.

In 1912 Higuchi reported similar positive results (143) using normal placenta, blood, embryonic skin, mammary gland and spleen as sources of the inoculum. Similarly, in 1919, Itami (150) showed the power of normal but embryonic liver to "induce resistance" to transplantable tumors.

In another direction inoculations were given to mice already bearing the transplanted tumor. Oser and Pribram (232) in 1913 found that the injection of spleen pulp into sarcoma-implanted rats sometimes resulted in shrinkage of the tumors and at other times brought the tumor growth to a standstill. The work of Bungeler (71) in 1932, using spleen implants in animals bearing the Ehrlich ascites adenocarcinoma or malignant chondroma, failed to change the development of the tumors. This showed the value of pulping the tissue over implanting whole tissue and raises the question of whether the spleen has some immunological role or simply contains inhibitors. Two years later Fischera (109) prepared extracts from foetal spleen and thymus and treated 300 patients, 100 of whom had inoperable cancer. At the end of 3 years regression was reported to have occurred in about 9% of the patients and the tumors had

become stationary in about 8%. The greater success of extracts may indicate these organs contain extractable inhibitors, but the immunological role of these organs does not permit one to exclude that their anti-tumor substances are not antibody-like.

About this time Fardon (104) separated out cell free extracts of normal tissues and with them found that mice could be rendered "resistant" by injections. He showed that the active principle, separate from the tissue pulp was water soluble. It was also not a protein fraction while the injection of a separate protein fraction increased the susceptibility of the mice to tumors. The non-protein nature of the active extract began to cast doubt on the "immunizing" function in making animals "resistant".

Domagk and Hackman (82) in 1935 also obtained results with cell free water extracts, from tumor and normal tissue, successful in making the animals "resistant" to the transplantable tumor. Seemingly paradoxically, in the case of established tumors, they found that large doses of the fraction stimulated the growth of the neoplasms. Thus in one fraction growth promoting activity existed along with an inducer of "resistance" or growth retarding principle. The "immunizing principle" was said to be labile and care had to be taken in preparing the extracts. Bauer et al. (32) in 1936 fractionated extracts of spleen tissue and inhibited tumor growth with those fractions soluble in alcohol. Purification of the alcohol soluble material with benzene doubled the activity of the extracts. This finding could have finally eliminated the idea of antigenic substances in these anti-neoplastic extracts, on the grounds that antigenic polymers were unlikely to remain soluble in benzene.

Water soluble beef spleen extracts were tested by Lewisohn (193) and reported in 1938. Subcutaneous injections of concentrated spleen

extract were given to mice bearing Sarcoma 180. Of the 281 treated sarcomas, 60% were absorbed and considered healed with no recurrence during a period of five months while only 8% of the 290 controls regressed spontaneously. It was reported that low concentrations of the extracts had great stimulatory effects on the tumors. A further example of this type of phenomenon was seen with injections of crude liver extract which caused leukocytosis in man (246) in 1933. In addition to the differences in growth due to a quantitative variation in tissue extract concentration the idea of tissue age was invoked by the finding of an inhibitor in adult tissue (291) in 1937. It appears difficult to assess tissue age or concentration effects in these extracts in terms of a growth control mechanism.

In assessing the action of the various tissue extracts some cell function was sought as a measurable parameter. Since Warburg in 1925 (330) reported that respiration of carcinoma was less than that of kidney and liver, respiration became the popular field of study and glucose metabolism the particular parameter. Bungeler (70) in 1930 found respiration of normal tissue of strains of mice resistant to a transplantable tumor was higher than that of mice susceptible. He also showed a spleen extract could stimulate respiration and depress gycolysis of tumors. Davis (79) 1937 confirmed that respiration is high in resistant mice while Schroder and Cook (280) confirmed in 1939 that extracts from normal tissue increased respiration and depressed both aerobic and anaerobic glycolysis of normal liver and tumor tissues. Similarly Sperti (295) described the respiration-stimulating power of beef spleen extract on a mouse sarcoma in 1941. Thus evidence indicated that agents which had the power to counteract tumor growth also affected glucose metabolism

of malignant cells. The difference in respiration between cancerous and normal tissues or between tissue from normal resistant or susceptible strains of mice was again shown in 1942 by finding alterations in the respiration of precancerous lesions (7).

Use of extracts of human spleen, and liver in the treatment of human malignancies was made with considerable success in 1946 (8) and 1950 (9). Preparations of protein and fat free water soluble extracts of tissues were injected intradermally around and under the lesions. Human epitheliomas were used as they were of low malignancy, localized and easily accessible. Only 1 of the 21 lesions treated failed to regress, 14 had had complete regression and disappeared. The last 6 were, at the time of reporting, still under treatment but had all regressed. Injections were also given at some distance from the lesion to test if they had tumor-specific inhibition at a distance rather than only a local physical action due to the solution on the lesion. Regression of the distant malignant area took place with no obvious clinical changes in the normal tissue at the site of injection. As a control, injection of glucose around the tumor failed to regress the tumor, so the extract's action was not as a sclerosing agent. It appeared that the more malignant lesions responded more readily to the treatment and again indicated by their difference in response that the action of the injections was not a purely physical effect.

Recognition of the presence of growth controlling factors in normal tissues was made in a review article 'The Biology Of Cancer' in 1957. The article states: (40)

> The nature of the environmental controls by which a healthy organism prevails upon its many cells to adopt and maintain a sociable way of life and which the cancer cells have to overcome (and) a knowledge of how these controls are affected, remain a

> major problem of current biology, but there is little doubt that chemical substances, humoral factors pass from organ to organ influencing the function and size of organs."

The discovery of growth inhibitors continued. In 1958 it was reported that serum and a liver homogenate from adult rats contained a factor which could inhibit the onset of mitosis in regenerating liver. Serum and liver homogenates from partially hepatectomized rats however showed no retarding effect (246). Apparently then, the liver produced a growth inhibitor as long as the liver cells were not dividing, but when called upon to regenerate new tissue the liver stopped making the inhibitor. Difficulties in this type of research could occur with the apparent contamination of extracts of normal tissues by 'growth promoting' factors (240). Alternately the same substances might act as inhibitors or promotors depending on the concentration, as mentioned above. Marshak(207) reported finding some stimulatory and some retarding fractions of liver, showing their effect on mitosis in regenerating liver. Likewise growth promoting and retarding factors were seen by Werner (336). The conditions under which stimulation or inhibition occurs following administration of the same type of material are as yet not fully understood, (one factor may be dosage), but the two opposite effects from fractions of the same organ are hard to explain from an immunological standpoint, although not perhaps impossible.

Further reports on tumor inhibitors confirmed their presence in normal tissues (134). Extracts of spleen and lung injected intraperitonally into rats and mice bearing ascites cells inhibited the tumor, while similar injections were not in any way toxic to normal animals used as a control. The factors were in lungs of both tumor bearing animals and normal animals. They were believed to be nuclear and not cytoplasmic

in origin. Such inhibitors in these studies were not found to be in the liver. However many other reports cited inhibitors especially in liver. Herbut and Kramer (141) showed complete regression of growth by extracts of guinea pig liver while marked but incomplete retardation of growth was induced by extracts of sheep, hog, and rabbit livers. Slight but inconsistent retardation was demonstrated with horse and bovine liver. The idea of immunity against cancerous cells had not entirely died however and a tumor-lethal system involving antibody complement in normal serum was reported (184) in 1960. At the same time liver extracts were being tested on cultured mammalian cells with some success in inhibiting their growth (198). Likewise filtrates of chick embryo (203) were reported to inhibit cells in vitro. Such in vitro assay systems proved useful to many investigators and demonstrated the effect of cerebrospinal fluid and serum on stopping cell growth (105). Also serum cytotoxic activity was shown in this system (106) but it was later reported that there were natural cytotoxic "antibodies" in blood which reacted with the mammalian cells (107), again suggesting an immunological reaction towards cancer. But in the use of tissue cultures there was no possibility of inducing 'resistance', or immunizing the cells. This technique could finally distinguish immunity through antigens from direct cell growth inhibition. With this clarification made, tissue extracts were being tested for their inhibitory rather than their "immunizing" activity. Adult connective tissue was said to contain an inhibitor (236) (237) (239) of fibroblasts in vitro. Activity was found in both dialysable and non-dialysable materials. The inhibition of tumors by these same materials was even considered to be quite specific (238). Two anti-tumor substances were purified and studied by Friedman et al. (121) in 1962.

More than one inhibitor was found in a variety of normal tissues by Bardos (25). Some inhibitors were said to be high molecular weight polypeptides from normal tissues (226) and, more specifically unfractionated preparations of histones from mammalian tissues were reported to be capable of inhibiting transplanted tumors (328). Other work on basic polypeptides from animal tissues indicated a strong inhibition of tumor cell culture (245). In spite of the large number of similar findings of naturally occurring inhibitors the chemical nature of the inhibitors has remained elusive, and very thinly investigated. The fact of their existence, however, has been established.

Accepting the idea that normal tissue proliferation might be regulated by humoral factors, it was supposed that leukemia was caused by an increase in a myeloproliferative factor (78) or by a decrease in an inhibitor which normally repressed leukopoesis (338). It could equally be assumed that equilibrium between different factors was disturbed. In 1936 Wiseman, Doan and Erf presumed the existence of a reciprocal relationship between the lymphoid and myeloid tissue (343). That is myelocytes control the growth of lymphocytes through some secreted factor, and vice versa. In 1943 Miller and Turner (221) demonstrated that there were indeed factors isolated from urine and serum which stimulated the myeloid tissue and separate factors which stimulated lymphoid tissue. They were said to be steroid-like principles which could be called myelokentric acids, and produced specific histological changes in the tissues in question. Given the principle from urine of patients with myeloid leukemia, animals showed organ changes similar to changes in myeloid leukemia. But given equal amounts of the principles from urine of myeloid and lymphoid leukemias a normal histological picture was seen.

Observations have been made that treatment of leukemic patients with blood transfusions sometimes produced a clinical remission and an improved blood picture which could not be completely explained (338). Sometimes a remission was also obtained following transfusion of fresh plasma (279). This consequently emphasized that normal plasma contained a substance which counteracted the proliferation of leukopoetic tissue and attempts were made to treat human and animal leukemia with various tissue extracts. Tremblay successfully treated different kinds of leukemia in man with fractions of myelocytic tissue, bone marrow, and of lymph node tissue(321). Miller et al. (220) treated eight patients with lymphoblastic leukemia with 'myelokentric acid' extracted from the urine of patients with chronic myelocytic leukemia. Partial remissions were observed. Thus myelocytic factors may have controlled lymphocyte growth.

The reciprocal relationship suggests lymphocytes can control myelocytes. Gerhlich (124) reported remission in acute leukemia following the intraperitoneal injection of guinea pig serum (168). Similarly some tissue extracts inhibited a transplanted myelocytic leukemia in rats (331), but other studies reported a rise coincidentally, in the number of normal leukocytes in blood. This leukocytosis was stimulated by a crude extract of liver (246). It was further studied (80) in relation to the resultant increased resistance against infections. Later investigations dealt with the effect of the liver fraction on the life span and development of 'spontaneous' leukemia in the high-leukemia AKA strain of mice. It had a protective effect against development of leukemia and permitted a prolonged life span (17). It thus had an indirect antimyeloblast activity through a specific growth stimulatory effect on mature leukocytes. Perhaps mature leukocytes could prevent the growth of immature cells.

Another example of a serum factor was the inhibiting activity of guinea pig serum on cell cultures (182). But this activity is related to its asparaginase content as seen by the effects of the asparaginase fraction on an ascites lymphosarcoma (144), (50). This enzyme, by depriving cells of asparagine, specifically caused cell death. It is not known if there was a further cytotoxic factor in serum besides this enzyme.

Liver, muscle and spleen tissues were continuing as targets of investigation (59). Druckrey et al. used several tissue homogenates to inhibit tumor growth (85). Their lung and spleen tissue extract also destroyed cancer cells in vitro (84), thus indicating a specific cell inhibition as opposed to an immunizing function. Such homogenates were reported to be responsible for microscopic damage specifically to the tumor cells (278) (297). In 1966 Sato reported the effect of skeletal muscle extracts in inhibiting Ehrlich Ascites tumor cells (272). Sugihara and Hori prepared carcinostatic substances from bovine liver (300) (149) and Watson used calf spleen extracts to inhibit spontaneous mouse tumors (333). Ting reported a pig liver substance with inhibitory powers (320) in 1969. As most of these endeavours do not lend themselves to adequate comparison but do suggest that normal tissues contain substances which inhibit cell division, an attempt was made to systematize the search for natural growth inhibitors. An organized investigation of many animal tissues was made and reported in 1968 (26). Of 1000 fractions from 28 different bovine and porcine tissues, antineoplastic activity was found in 14 fractions, cell culture cytotoxicity was found in 8 fractions from liver, 1 from lung, and 2 from the pineal gland. Antineoplastic activity was also reported in clam liver extracts (195). These extracts were

particularly capable of inhibiting viral oncogenesis (194). In such studies the effect of asparaginase, methotrexate (4-aminomethylfolic acid, a folic acid antagonist), and liver extract were compared on Rous Sarcoma Virus focus formation in vitro, and the extract demonstrated strong inhibition.

In assessing the above reports of anti-growth factors from normal tissues one can at least conclude that such factors do exist. The idea that normal tissues can control their own growth through chemical mediators is frequently supported. These mediators appear capable of regressing not only animal but human cancers, with no reported harmful effects on normal tissues. Even virus proliferation is inhibited in some cases. The chemical nature of the inhibitors appears, on the surface, to be diverse. Depending on the report, the factors could be stable, labile, dialysable, nondialysable, peptidal, or non-protein. If it is accepted that there are common mechanisms of growth and cell division in all cells, regardless of the tissue, it might seem plausible that only one inhibiting principle exists in all tissues. In any case the evidence over sixty years calls for a serious chemical search for the nature of these inhibitors. Not only is there evidence that they exist but a unifying concept was elaborated to explain their behaviour. Osgoode postulated in 1957 (233) that malignancy is due

> "not to the presence of something but rather the absence of something. The fundamental alteration is any genetic change leading to lack of any enzyme system necessary for a full life span of the differentiating cell of a series. This chemically mature differentiating cell produces inhibitors of cell division. Lack of the inhibitor permits the emergence of the capacity for unlimited and uncontrolled growth that is present in the unicellular organism and has been retained in the early dividing cell of every fundamental cell type in at least some stages of postnatal life. This concept does not displace current theories of the etiology of malignancy but rather shows how all may be correct and have in common the fact that they predispose to

> the development of some change in the cell that leads to a shortened life span and hence to the lack of growth inhibitors".

This theory explains that the balance between cell production and cell destruction exists as a result of a negative feedback. In tissues of adult animals the phenomenon of balance between cell production and destruction has been known for so long that it is easy to take it for granted and ignore the problem of how these cells are capable of maintaining the balance. For at least 30 years it has been obvious that a disorder in the regulation of cell production is an essential feature in carcinogenesis: the growth of a tumor is naturally only possible when cell production exceeds cell losses (34). Again this implies that in in neoplasia the mechanism maintaining the balance is damaged, destroyed or not attained.

The concept of a negative feedback applied to tissues suggests that the growth of individual cells depends on a chemical signal: an inhibitor, produced by mature cells. As destruction of mature cells takes place the inhibitor disappears. Those parts of the immature cell responsible for cell proliferation recognize the intensity of the signal and respond to a diminished signal with accelerated cell production. New mature cells producing the inhibitor maintain a high intensity of the signal as long as they live. It is necessarily assumed that the signal's duration is usually short.

B. Chalones.

Is there experimental evidence of such a signal? Are the inhibitors in extracts of liver, spleen etc., examples of these signals? It has been shown recently that compounds released by cells of certain tissues inhibit the mitotic activity of cells of homologous tissue (61) (69) (46).

The term 'Chalone' has been used to describe such compounds. A Chalone is the opposite of a hormone in that it curbs activity of its target. Some of the properties of chalones are that they inhibit mitotic activity both in vivo and in vitro, their action is rapid and reversible, tissue-specific but not species specific, and that in the case of some they require stress hormones (eg: Adrenaline) as co-factors (64)(65)(68) (265) (266). When the problems of neoplasia and the normal feedback control mechanism are considered in terms of chalones it is possible that changes in their structure, in their rate of formation, in the mechanism of their intercellular or intracellular transport, or in the reactivity of the cells, could give rise to neoplasia (62). The action of chalones has so far been studied in three malignant tumors: epidermal carcinoma, melanoma, and leukemia. (66) (67) (68) (267) (268). In all these cases it was found that the tumor cells continued to produce homologous chalone but that their chalone content was only a fraction of that in normal cells. Further, when the chalone content was increased by exogenous means it was found that mitotic activity was inhibited in the malignant cells as well. The first few attempts to treat a malignant tumor with homologous chalone which were carried out (222) revealed that the tumor growth slowed down, stopped, and completely regressed. In both mice and hamster, melanomas disappeared in all cases after chalone treatment of only five days duration even though in many cases the tumor recurred later. Similar results were obtained with chloroleukemia tumors in rats (269) treated with granulocytic chalone; in this case the inhibition was permanent. While the tumor cells were killed by chalone treatment the normal cells were not destroyed. Normal granulocyte production recovered, apparently because the pluripotential stem cells

did not react with the granulocytic chalone (127).

Bullough has summarized the nature of chalones in general (63). In short they are proteins or glycoproteins which control mitotic activity by a negative feedback mechanism. Most of the chalones have a molecular weight of about 30,000 to 40,000 (46). However the granulocytic chalone, like the erythrocytic chalone has a low molecular weight (about 3000) but is, nevertheless, a glycoprotein (183) (265). Recently a liver chalone was isolated with a weight of similar size (326a).

The rate of synthesis of a chalone is probably constant (63). It is known that chalone molecules are present in urine (63) therefore their rate of excretion must be important to the concentration of a chalone in the body; the latter should depend on the rate of synthesis and the rate of excretion or destruction. The concentration within a tissue cell will be influenced by the rate at which the chalone is lost across the cell membrane. In the rabbit epidermal carcinoma (66) and the rat granulocytic leukemia (267) the tumor cells continued to produce the chalone of their tissue of origin but the intracellular chalone concentration was one tenth or less of normal. In the leukemia it was shown that the low chalone content was primarily due to an increased rate of chalone loss through the cell membrane. Exogenously administered chalone actively inhibited the mitotic activity of the tumors showing that the cells retain their ability to respond by mitotic inhibition when chalone concentration is restored to normal. Bullough admits that as mitosis itself can hardly be tissue specific, part of the chalone molecule which is anti-mitotic might be common to all chalones. Such a composition could explain why researchers for fifty years found inhibitory activity in extracts, some containing protein and some

definitely without protein. These latter may have been that fragment of a chalone which had the antimitotic activity freed from the protein carrier specific for a certain cell type. Dr. I. Hendeson, a surgeon in Montreal, following a procedure for extracting chalones, ended up with a preparation resembling a ketoaldehyde (140), a compound known to inhibit growth.

C. Retine

Has there been any work parallel to this type of tissue extraction which has delineated further the chemical nature of the tissue inhibitors? One laboratory in particular has pursued this type of inhibitor to the point of partly identifying the inhibitor. The work of Albert Szent-Györgyi and his associate Dr. L.Egyud has illustrated a tenacious search over the last 15 years for the inhibitor.

Their work began with the observation that extracts of thymus gland were coloured more yellow than could be explained by their flavine mononucleotide content (302) (303). Testing these extracts on malignant growth they found that the growth slowed down or stopped altogether (309). The extracts sometimes showed that they contained two active substances, the one promoting, the other inhibiting, malignant growth, and the result depended on their balance. This experience was not unlike that of the workers cited above who found stimulatory and inhibitory factors in their tissues, depending on the dosage given to the test animal. The retarding substance of Szent-Györgyi was called 'retine', the promotor 'promine'. Promine fractions also contained a factor which was believed to sterilize mice (135).

Retine was also found in tendon and aorta, and thus seemed to be a general tissue constituent (311). Tendon, big blood vessels and muscles are three tissues which show malignant growth least frequently. Parshley also found inhibitors in these tissues (236). The idea of using this

natural growth inhibitor in cancer therapy was suggested in 1963 (310) while all that was known chemically was that it was a small molecule, decomposed at room temperature in a week, and was more sensitive to alkali than to acid. No harmful side effects in the recipient were found. In the hope that urine might reflect the ratio of promine and retine in the body, Szent-Györgyi tested urine of children. Certain fractions did have retine and later, young adult urine was examined and found also to contain retine. Concentrated urine samples gave a purified product of retine which on dialysis and Sephadex chromatography displayed a molecular weight in the range between 400 and 1000 (136).

Extracts of clam were found to have retine which inhibited Sarcoma 180 and Krebs 2 carcinoma. Sephadex separation of this material indicated a weight in a range less than 10,000 (304). Retine behaved sometimes as a colloid macro-molecule, others as a crystalloid of small weight. Some extracts were stable, some deteriorated rapidly. It was shown that retine was indeed small but bound in vivo to a colloid from which it could be released.

Those retine samples which showed small weight and low boiling point, on acid treatment changed to an even lower boiling point. The U.V. absorption at 273 nm was greatly increased. The product became very toxic though maintained its anti-tumor activity. This change was thought to be due to a splitting off of some group from the molecule. The 'split' retine was less soluble in water. Infrared (I.R.) studies indicated a ketone.

By steam distillation the highly purified preparation indicated the presence of a ketone and an aldehyde while the low boiling point indicated a very small molecule. The simplest ketone aldehyde is methyl glyoxal

CH_3-CO-CHO, and has a U.V. absorption peak at 272 nm which showed a rise in absorbance with acid or alkali. The carcinostatic action of methyl glyoxal derivatives had been known at least since 1953 (22). Some analogues, eg: Kethoxal, 2-Keto-3-ethoxybutyraldehyde (118), have been on the market for treating malignant diseases. One derivative (hydroxymethyl glyoxal bis guanylhydrazone) was found in some concentrations to be an inhibitor, in others a promoter of growth (117). Methyl glyoxal was tested on fertilized sea urchin eggs and at 10^{-4}M inhibited mitosis (304) but left the spindle intact. Like retine, methyl glyoxal was toxic to tissue culture cells at 10^{-3}M to 10^{-4}M. The ketone aldehyde is a most reactive grouping; it easily undergoes isomerization or polymerization and makes the molecule unstable. This could account for the difficulty in isolating retine. But retine was said to be not identical with methyl glyoxal; it was more active (304).

Retine showed a U.V. absorbance at 272 nm in water which shifted to 292 nm when made alkaline (91), a characteristic of ketoaldehydes due to formation of the enolate anion. The I.R. indicated, besides ketone and aldehyde carbonyls, the presence of alkenes (unsaturated carbon bonds) and possibly a hydroxyl band around 3300 cm^{-1}.

As a dinitrophenylhydrazine (DNPH) derivative was made which had no biological activity it indicated that the carbonyl groups were necessary to the activity of the molecule (308). The infrared spectrum of the DNPH derivative also showed the band near 3362 cm^{-1} (possibly -OH) and a 1645 cm^{-1} band probably of unsaturated carbon, but the interpretation of the spectrum as a derivative of carbon suboxide, was completely incorrect as given by Marmasse (206).

Ketoaldehydes inhibited E. coli growth (96). The reversal of this inhibition by cysteine exemplified that ketoaldehydes react with -SH

groups (96). The incorporation of radioactive precursors into DNA, RNA, and protein showed that methyl glyoxal inhibited proliferation of E. coli by interfering with protein synthesis primarily. Ninety-four percent of aminoacid incorporation was stopped, 50% of Thymine incorporation was stopped, then 25% of Uracil incorporation was stopped. The protein synthesis was reactivated by the addition of -SH compounds, and nucleic acid synthesis followed (97). The antagonism of thiols towards α ketoaldehydes was investigated more closely (94). The antagonism was found to depend on the number of -SH groups and their relative position. For instance two were necessary for strong inhibition, if they were vicinal as in dithioglycol. Cysteine also was active with its vicinal -SH and -NH groups but cysteamine was not active implicating an important specific role for the - COOH group of cysteine.

Alpha ketoaldehydes were more inhibitory of cell division than aldehydes or ketones alone (101). The duration of the inhibition depended on the length of the molecule: ethyl and propyl glyoxal (containing 4 and 5 carbons respectively) showed a longer inhibition than methyl glyoxal (3 C atoms). One anomaly was that decyl glyoxal which contains 12 C atoms slightly promoted growth, and occasionally nonyl (C_{11}) and undecylglyoxal (C_{13}) also promoted. Methyl glyoxal was also shown to inhibit, at 10^{-3}M, the capacity of E. coli cells to produce mature phage (100).

The isolation of retine from liver posed the problem of contamination of the fractions with a glucose derivative (95) (113) (305) which was made from reaction of glucose (from glycogen) with amino acids (from protein breakdown) (235) (23). Here an artifact was produced resembling a ketoaldehyde. A liver protein fraction inhibited DNA and RNA synthesis more than protein synthesis indicating it had not exactly the same type

of inhibition as had methyl glyoxal (234) or retine.

The efforts of Szent-Györgyi and his associates have led to the assertion that ketoaldehydes are the animal's natural growth controlling agents. They are reactive but they can be stabilized on a carrier presumably protein in nature. They hold back cells from dividing and can be destroyed by the enzyme glyoxalase utilizing glutathione (GSH) as co-enzyme. Normally the glyoxalase is considered to be compartmentalized while the ketoaldehyde is at large inhibiting cell division. Upon injury the enzyme is liberated from its compartment, ketoaldehyde is destroyed, and cell division proceeds to replace the damaged tissue (307).

II. Carbonyls Inhibit Tumor Growth.

It is not surprising in retrospect that division is stopped by ketoaldehydes and their derivatives, or similar structures which may react with important cell groups such as -SH or $-NH_2$. That -SH fluctuations are important to cell division (295) has been well known since Rapkine's discovery (252). The same G. Domagk who, in 1935 (82) used cell free water extracts with anti-tumor activity, found in 1946 (81) that thiosemicarbazones derived from acetamidobenzaldehyde and thiosemicarbazide, or, paradimethylamino benzaldehyde and the same reagent, possessed significant anti-tumor activity. Derivatives of aldehydes were tested for antigrowth powers on a larger scale: in 1953 derivatives of methyl glyoxal were found to have anti-tumor activities (22). A number of these derivatives were further explored. Hydroxymethyl glyoxal bis guanylhydrazine (117), glyoxal bis thiosemicarbazone (119), Kethoxal (118), kethoxal thiosemicarbazone (244), glyoxal bis guanylhydrazone (116), and dihydrazones (120) (122), all with anti-neoplastic success. Derivatives revert to yield the free carbonyl when in the cell (286). As a free dicarbonyl (not a derivative) the ketoaldehyde methyl glyoxal was found to suppress leukemia in mice (12). Both methyl glyoxal and glyceraldehyde, two components of known metabolic pathways related to the 3-carbon stage of glycolysis were found to inhibit cancer in mice. Mixtures of the two compounds were more active than when used alone. Many different cancers were inhibited (13) (14) (15) (16). Recently more evidence of the anti-neoplastic action of methyl glyoxal was given by Jerzykowski et al.(155). They reported that intraperitoneal injections of the ketoaldehyde cured or protected mice bearing Ehrlich Ascites cells, or rats with Yoshida Ascites sarcoma, or hamsters with Kirkman-Robbins hepatoma. Given subcutaneously

in the region of solid tumor growth methyl glyoxal showed a powerful antineoplastic effect.

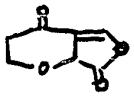
That their action is against -SH or -NH- groups in the cell is supported by Knock (173) who claims to have observed regression of human tumors by agents which bound to -SH groups in the tumor cells. The electrophilic activity of the dicarbonyl is due to the deficiency of electrons on the two adjacent carbonyl carbons. A similar electrophilic activity could exist on an α,β unsaturated aldehyde C=CH-CHO, and indeed 4-hydroxy-2-alkenals R — CH(OH)- CH=CH-CHO were found by Schauenstein's group to inhibit malignant cells (101) (102). These compounds came from autoxidation of unsaturated fatty acid esters in water (275). They inhibited DNA and RNA synthesis of Ehrlich Ascites tumor cells (276) and specifically inhibited the tumor cell growth <u>in vivo</u>, especially 4-hydroxy-pentenal CH_3-CH(OH)-CH=CH-CHO. The 4-hydroxy group was found essential for the action, as without it no activity was recorded. As an enal would be quite unspecifically reactive, the 4-OH group, which provides the specificity, could form a cyclic hemiacetal with the aldehyde protecting it until it reached its target. It is this tautomeric form which could be responsible for the specific activity. In summary tumors have been treated successfully with electrophilic compounds of a carbonyl nature.

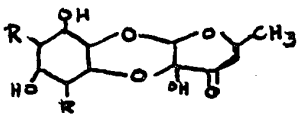
III. Carbonyls and Derivatives Inhibit Viruses.

Ketoaldehydes not only inhibit tumors but have been shown also to inhibit viruses. Derivatives of benzaldehyde, an aldehyde, and thiosemicarbazone had been reported as antiviral agents (132). Other carbonyl adducts like isatin-3-thiosemicarbazone worked against pox viruses and polio (337). Thiosemicarbazide derivatives of other aldehydes like nicotinaldehyde, isonicotinaldehyde and 2- and 3-thiophenaldehydes have also shown antiviral activity (315). 4-bromo-3-methylisothiazole-5-carbox-aldehyde thiosemicarbazone protected mice against neuro-vaccinia virus (Cf. 337). Thiosemicarbazones derived from substituted pyrrolidine-2, 3-diones (diketones) protected against influenza in mice (Cf. 337). 2-keto-3-ethoxybutyraldehyde (Kethoxal) has been shown to have antiviral activity against PR-8 influenza and Newcastle disease viruses (324) (215). Aliphatic glyoxals, α-hydroxyaldehydes and related structures were shown to inhibit virus proliferation in 1957 (318) (319). In 1961 Kethoxal was compared to the toxic aldehyde formaldehyde against the DNA viruses: virulent polio virus and vaccinia virus. The reactivities of the two agents were similarly inhibitory. Against the RNA containing Newcastle virus Kethoxal was more active than formaldehyde (325). Derivatives of ketoaldehydes inhibited influenza virus (201) arbovirus (6) and encephalomyocarditis (314). Antiviral compounds were found among the aminoacethydrazones of aromatic α-ketoaldehydes by Massarini et al. (210). Kuchler et al. (179) reported that methyl glyoxal bis guanylhydrazone had antiviral activity. Kethoxal inhibited Herpes simplex virus (323) and other viruses (256). Antiviral activity was found in oxidized polyamines which would be unsaturated aldehydes, and in simple aldehydes (178). If ketoaldehydes could be expected to be the unknown growth inhibitors in

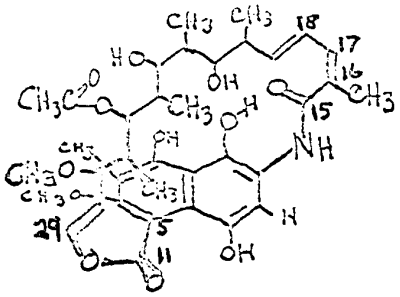
mammalian tissue extracts it is interesting to see activities common to ketoaldehydes appear in clam liver extract. The latter inhibited Rous sarcoma virus (194) adenovirus (197) and others (196), as well as inhibiting neoplasms (195).

IV. Carbonyls Inhibit Bacteria and Animal Cells.

In 1961 Seifter et al. reported testing 200 carbonyl compounds and their derivatives against 21 micro-organisms. For general antimicrobial activity, among those tested, compounds related to pyridoxal and menadione (Vit.K) were most active when used as an adduct with a carbonyl reagent (286). The most useful carbonyl reagents were those possessing ring structures which act as electron donors to the reactive nitrogen HN=N-⬡ . All adducts had been expected to revert and yield the free carbonyl inside the cell where the carbonyl would react with the target (286). Aldehydes can also be protected from reaction by having hydroxy groups form hemiacetals with them. Internal hemiacetals for example occur in reducing sugars. If a hemiacetal is oxidized a lactone results. The antibiotic Patulin  was said, in 1943, to have a great capacity for minimizing the effect of the common cold by inhibiting bacteria (41). Like ∝ angelica lactone it has an unsaturated lactone grouping which reacts with -SH groups by forming a thiol ester and a carbonyl (137). Patulin also has an ∝,β -unsaturated ketone, and an ∝ -alkoxy ketone, both capable of inhibitory reactivity.

Actinospectacin (147) (341)  contains a hemiacetal which would readily open depending on ionic or pH conditions, to provide a diketone. Actinospectacin is a new antibiotic used until now only in veterinary cases, except experimentally in humans.

Rifamycins are compounds consisting of a chromophoric naphthoquinone ring which is spanned by a long aliphatic bridge (335). Rifamycin S V has the following structure:



A structure similar to angelica appears with carbons 28, 29, 11, and 5. An α,β - γ,δ unsaturated carbonyl exists from carbon 15 to 20.

Both structures are capable of reacting with nucleophilic groups. Rifamycins are antibiotics and growth inhibitors, one of whose reactions is binding the RNA polymerase. Recent evidence suggests that these derivatives affect the reverse transcriptase (130a). Rifamycin is particularly successful against m.tuberculosis. Other successful arrest of tubercular activity occurred with adducts of pyridine-4-aldehyde and pyridine carboxylic acids. Other methods of concealing the aldehyde group have been by the use of thiosemicarbazones (81) as mentioned previously.

Without an adduct on the carbonyl the anti-bacterial and antiviral activity of aldehydes has been recognized just as well, and the use of glyoxal as potential blood sterilizing agents was recommended in 1956 (324). The action of aldehydes, ketones and ketoaldehydes on E. coli was studied by Egyud (93), as mentioned above. He reported that the aldehydes and 2-ketones, although they moderately inhibited the bacteria, showed a transient effect. Strong inhibitory action was obtained only by those compounds where a 1, 2 dicarbonyl was present. The action was presumed to be against sulfhydryl groups, since cysteine reversed the inhibition (308) (97). An exception to the activity of single ketones was methyl vinyl ketone which showed a strong blocking effect on E. coli proliferation (93). This compound has an α,β double bond which activates the carbonyl carbon and encourages a strong electrophilic attraction to -SH compounds. A

further example of an α,β unsaturated carbonyl is the compound 4-hydroxy-pentenal which has recently been reported as a powerful cytotoxic compound. Its effect was most readily observed on actively proliferating mammalian cells but less so on non-dividing cells (274). Since -SH groups are plentiful during cell proliferation (211) (252) one may suspect reaction of the α,β unsaturated aldehyde with them as has been suggested (72) (275). Evidence that carbonyls generally react with -SH groups has long been established (253) (281) (282). In the studies with 4-hydroxypentenal cytoplasmic degeneration was evident before any nuclear damage was observed. However it might have been expected that antisulfhydryl compounds react primarily with membrane -SH groups which are diverse and have specific reactivities towards various reagents (37). Whether the cytoplasmic degeneration is secondary to the reaction of membrane -SH groups with aldehydes or ketoaldehydes is not known. Examples have shown that methyl glyoxal (3 mM), selectively inhibited synthesis of DNA 90%, protein synthesis 82%, and RNA synthesis 24%, in sheep lymphocytes. It inhibited DNA synthesis 71%, protein synthesis 85% and RNA synthesis not at all in human epithelial cells; it inhibited both DNA and protein synthesis about 50% in human amnion cells and RNA not at all (340). This action of methyl glyoxal contrasts with that of cortisol-21-aldehyde, another α-ketoaldehyde which suppressed RNA synthesis as well in these cells. Neither cortisol aldehyde (0.5 mM) nor methyl glyoxal (3 mM) uncoupled oxidative phosphorylation in mitochondria and this implies that both these ketoaldehydes were selectively inhibiting polymer biosynthesis. Diacetyl (1 mM), the simplest diketo analogue of methyl glyoxal, had no effect on thymine incorporation by the lymphoid and epithelial cells but actually stimulated uridine incorporation by these cells. Klamerth (172) showed that glyoxal was cytotoxic for fibroblasts

in culture by blocking DNA and protein synthesis primarily. The synthesis of rapidly labelled RNA was not inhibited except after prolonged incubation. Gregg (127), using methyl and propyl glyoxal, completely inhibited cell division in cultured lymphoma cells (L5178Y); the inhibition was reversed by equimolar cysteine. Protein synthesis was inhibited 95% and DNA 60% but RNA synthesis was unaffected until much later. The derivative Kethoxal bis thiosemicarbazone, a 'cytostatic agent', acted primarily on DNA synthesis (49). Addition of methyl glyoxal or Kethoxal to synchronized cells in either G_1 or S or G_2 stages of the cell cycle, was found to inhibit their subsequent division (273). Likewise in asynchronous cultures, mitotic figures rapidly disappeared with treatment. Cells treated in G_1 remained strongly inhibited 18 hrs after addition of inhibitor. However cells treated in S phase appeared to be less sensitive and showed reversible inhibition. Cells in G_2 were strongly blocked. Both inhibitors stopped protein synthesis in S and G_2. DNA synthesis during S was moderately inhibited while RNA synthesis was not much affected.

In view of all these findings it would appear that this class of compound, the ketoaldehyde, does not act as a specific mitotic blocking agent but can inhibit at any point in the cell cycle by interfering in different ways with metabolic processes especially protein synthesis. The ketoaldehydes curb the proliferation of tumors, viruses, bacteria, and mammalian cells, and may play a role in normal tissue homeostasis. For this they would presumably have to be stabilized in some manner or be constantly produced. That they react with nucleophilic groups makes it important to know which nucleophilic target(s) in particular is responsible for proliferation.

V. Carbonyls and Respiration.

It will be recalled that the anti-tumor activity of extracts of liver and spleen was said also to depress glycolysis and stimulate respiration. That respiration was not lowered yet tumor proliferation decreased is an interesting observation which finds a parallel in the action of a ketoaldehyde. Whitehouse (340) reported no uncoupling of oxidative phosphorylation in animal cells, at concentrations of methyl glyoxal which inhibited polymer synthesis. Egyud and Szent-Gyorgyi reported that muscle contraction and respiration, both of which depend on -SH groups, were not inhibited by concentrations of methyl glyoxal ten times higher than those needed to stop cell proliferation (96) (97). Apparently dosage is a question here. The effect of methyl glyoxal on inhibiting respiration in a variety of tissues was reported in 1932 (170). By 1950 methyl glyoxal was shown to inhibit succinic dehydrogenase (180). Aldehydes in general were found to inhibit mitochondrial respiration as reported by Kiessling (169). Glyceraldehyde in particular, a precursor of methyl glyoxal, has had a long history of inhibiting respiration (218). Lardy et al. in 1950 explained that by blocking -SH groups glyceraldehyde inhibited glycolysis (186), and one year later Needham, Siminovitch, and Rapkine reported similar results indicating that glyceraldehyde formed methyl glyoxal non-enzymically, inhibited triosephosphate dehydrogenase directly (228), and reversed the inhibition in the presence of cysteine. Glyceraldehyde was also said to inhibit amino acid incorporation into protein of a hepatoma (130) and to inhibit nucleoside incorporation into DNA and RNA of an ascites tumor (231).

The role of glyceraldehyde as an inhibitor is interesting because a simple dehydration, which is easily catalysed by phosphate anion, (257)

can occur and yield a stronger inhibitor. Glyceraldehyde $CH_2OH-CHOH-CHO$ can lose one mole of water to form methyl glyoxal $CH_3-CO-CHO$. Such a non-enzymic reaction can occur more readily than is usually believed and will be discussed below.

VI. Accounting For Ketoaldehyde Production.

Dihydroxyacetone was discovered to be a good electron donor especially in alkaline conditions (306). This would encourage its reaction under aerobic conditions as oxygen is an electron acceptor. Dihydroxyacetone is interconvertible with glyceraldehyde. Besides phosphate ion, acid can catalyse the dehydration of glyceraldehyde or dihydroxyacetone, and methyl glyoxal results. The acid-catalysed dehydration of β-OH carbonyls yields α,β unsaturated carbonyls and is called beta elimination. Both the latter and ketoaldehydes are compounds reactive with nucleophilic groups. Should an α,β unsaturated ketone become oxidized to the ozonide and be decomposed by water a ketoaldehyde results (129). Grignard et al. (129) cite many other references to the non-enzymic production of ketoaldehydes. In 1898 Pinkus produced methyl glyoxal from a hexose using alkali. In 1910 Harries produced it from the ozonide of mesityl oxide with water. Fernbach reported it produced from sunlight on a hexose in 1914. Neuberg with hot ammonia on hexose (1915) and dilute H_2SO_4 on glyceraldehyde (1917). Fischler obtained it with Na_2Sulfite on a hexose in 1926. Riley (258) in 1932 introduced the Selenium dioxide oxidation of a monocarbonyl which produces a dicarbonyl. All these reactions indicate facility in producing the ketoaldehyde by simple methods of exciting the electrons for reactivity.

It was observed in 1937 that glucose, on losing one mole of water gave a deoxyosone R-CHOH-CHOH-CHO $\rightarrow$ R-CH_2-CO-CHO + H_2O. Extracts of Aspergillus parasiticus were (47) instrumental in this conversion but enzymes are not necessary for it to occur. The same year Needham and Lehman showed how glyceraldehyde was transformed into methyl glyoxal(227) non-enzymically in the presence of lysine and phosphates.

Ketoaldehydes are formed in the non-enzymic browning reaction (35) of fruit and in milk (329) (159). In these, oxidation seems to play a role and the ketoaldehyde reacts with proteins to produce the brown colour. Glucose or any aldose may react with an amino acid (ag: glycine) to give a glycosylamine derivative. This undergoes slowly and irreversibly the Amadori rearrangement to give D-fructose-glycine. These are typical reactions of amines with aldoses and are generally accepted as the first steps in the "Mailard Reaction" or non-enzymic browning (10) (11). The subsequent reactions remove the amino adduct leaving a 2-keto and 3-deoxy aldose (113). Sugars and amines yielded dicarbonyls in the studies of Hodge et al. (146) and the browning of glucose-lysine solution was said to be catalysed by trace metals (205). Markuze studied here the effect of oxygen, nitrogen and light on a glucose-lysine solution in phosphate buffer. Iron or copper accelerated browning while tin or manganese decreased browning. Lead had no effect. Evidently a redox change in the metal was accompanied by the browning, which is simultaneous with the dehydration of an aldose. An analogous reaction, dehydration coupled with a redox change, was discovered by Gabel. He was able to polymerize monophosphate, non-enzymically, that is to make phosphoric anhydride bonds, in dilute aqueous solution with the simultaneous oxidation of $Na_2S_2O_4$ (123). As oxidation is an electron exchange possibly phosphorylation of ADP to ATP accompanied by phosphoric anhydride bond formation, is a related phenomenon.

The formation of methyl glyoxal from glyceraldehyde can be catalysed by copper salts (129). The 'catalysis' of this reaction by lysine and phosphate was reported by Bonsignore et al. (48) and by Needham (227). The former group suggested that as lysine is present in the active site of

many enzymes it may have been the primordial catalyst.

Methyl glyoxal can be formed enzymically from amino acetone (99). Amino acetone can be made enzymically from threonine (126). Amino ketone biosynthesis was reported by Urata (326) and pathways involving threonine were studied by Willetts (342), especially enzymes acting on methyl glyoxal. Acetoacetate was enzymically converted to methyl glyoxal (224). Besides existing on normal metabolic routes, dicarbonyls have been found as odour components in the scent of stink bugs. In this material, besides hexanal, hexenal, octenal, and decenal, there was found an unidentified dicarbonyl compound (332). Dicarbonyls appeared in concentrates of aromatic substances from fruit (247) in apple aroma (176) and in apple and grape juices (175); no enzymic involvement was suspected in their appearance.

The enals mentioned in stink bug scent recall the enals with anti-growth powers of Schauenstein (275). These latter compounds came by autooxidation of unsaturated esters of fatty acids. Again a non-enzymic function produced growth-controlling substances, and their production depended on the oxidation state of the system. Fatty aldehydes, when rendered in a cyclic acetal form (250) proved to be quite stable. An acetal or hemiacetal protects an aldehyde from being indiscriminately reactive. The presence of the 4-OH group on Schauenstein's molecule allows cyclic hemiacetal formation with subsequent stabilization and this group was essential to the anti-growth activity.

To explain many of the above-cited reactions one needs to understand how the oxidation state may bring about non-enzymic production of ketoaldehydes. The processes may involve peroxidation and possibly free radical reactions. Hydrogen peroxide is produced in the cell during

metabolism and hydroperoxides R-O-O-H are produced from the oxidation of the unsaturated fatty acids present in lipids. Hydroperoxides can react with unsaturated groups:

$$R\text{-}O\text{-}O\text{-}H + 2\ CH_2{=}CHR' \longrightarrow R\text{-}O\text{-}CH_2\text{-}\dot{C}HR' + HO\text{-}CH_2\text{-}\dot{C}HR'$$

In the context of lipid peroxidation, hydroperoxide groups are produced in the same molecules that are unsaturated and this would lead to a significant increase in the rate of radical formation (249) (292).

There are two mechanisms for the decomposition of peroxidic compounds that are much faster than the slow unimolecular homolysis. They are molecule-induced homolysis (MIH), and One-electron redox reactions. The peroxide reaction with unsaturated groups is one MIH type. Another MIH reaction involves hydrogen donation from certain stable compounds to olefins. $R\text{-}SH + R\text{-}CH{=}CH_2 \longrightarrow R\text{-}\dot{S} + R\text{-}\dot{C}H\text{-}CH_3$

Oxygen reacts spontaneously with olefins. Poly peroxides are

$$O_2 + R\text{-}CH{=}CH_2 \longrightarrow \underset{\bullet}{R}\underset{\displaystyle O\text{-}O\text{-}}{\overset{\displaystyle |}{C}}H\text{-}CH_2$$

produced $(\text{-}CRH\text{-}CH_2\text{-}O\text{-}O\text{-})$. SO_2 gives an analogous reaction

$$O{=}S{=}O + R\text{-}CH{=}CH_2 \longrightarrow R\text{-}\underset{\displaystyle -O\text{-}S\text{-}O}{\overset{\displaystyle |}{\dot{C}}}H\ \text{-}CH_2\text{-}$$

and this accompanies a change in oxidation number of the sulfur.

One-electron redox reactions play a significant role (249) in the chemistry of cells and they are another way the oxidation state may bring about the non-enzymic reactions cited earlier for the production of ketoaldehydes. The reactions involve metals; transition metals catalyse decomposition of peroxidic compounds. Cellular fluids contain iron, copper and other transition metal ions. Numerous studies have shown the importance of transition metals in lipid peroxidation or on sulfhydryl oxidation both <u>in vivo</u> and <u>in vitro</u>. Fractions of tissue homogenates

spontaneously autooxidize if metal catalysts such as iron are added, and the oxygen uptake is directly related to the concentration of iron. Ferrous iron for example reacts with organic hydroperoxide $R\text{-}O\text{-}O\text{-}H + Fe^{+2} \longrightarrow R\text{-}\dot{O} + HO^{-} + Fe^{+3}$ and changes its oxidation number. Products of this reaction can initiate dehydration of vicinal hydroxyl groups as in glyceraldehyde.

Non-enzymic dehydration reactions seem to be important for producing the ketoaldehydes. In addition the non-enzymic reactions seem to be controlled by the redox state of the cell so that in the absence of oxidizing conditions ketoaldehydes may not be produced. If ketoaldehydes are expected to inhibit cell division then their absence permits cell division. In the absence of oxygen cells normally glycolize to gain energy. For example the epithelial cells lining the gut operate mainly on energy from glycolysis (212), adapted to do this, it is argued, because the pO_2 in the tissue of the gut lining is extremely low. This is especially true when the blood vessels could be constricted as during 'fight or flight' reactions. Other cells which operate mainly on glycolysis are tumor cells. Both tumor cells and epithelial cells of the gut undergo constant cell division. Lewis et al. (192) found an apparent deficiency in tumors of the 'widely distributed compound' methyl glyoxal.

Another instance where the redox state of the cell is involved with ketoaldehydes is the question of the glyoxalase enzyme and its co-enzyme glutathione (GSH). Their presence in all tissues suggests nature's expectation that the ketoaldehyde will be commonly produced and must be disposed of. But reduced glutathione (GSH) concentrations depend on the redox state of the cell since -SH is highly susceptible to direct oxidation. Redox changes are reflected by GSH fluctuations (211). With

prevailing conditions which keep GSH reduced the enzyme can eliminate ketoaldehydes from the cell. If GSH is oxidized to GSSG the latter can be reduced by $NADPH_2$ and the glutathione reductase. The pentose phosphate oxidative shunt furnishes the cell with $NADPH_2$ and this pathway is said to contribute greatly to metabolism of cancer cells in contrast to normal cells (cf. 173). The shunt is also said to be of greater importance in fetal than in adult heart (75). Other products of the shunt are fructose-6-phosphate and glyceraldehyde-3-phosphate which may be further metabolized by glycolysis as long as the glyceraldehyde-3-phosphate dehydrogenase is reduced by GSH, which in turn is maintained by the production of $NADPH_2$. Thus metabolism is controlled by the redox state, and some way cell division is closely related.

The two triose phosphates are interconvertible by an isomerase. They can either undergo oxidation by glyceraldehyde-3-phosphate dehydrogenase and NAD, or they can be reduced by glycerophosphate dehydrogenase and $NADH_2$ (75). Thus a B vitamin is essential for the breakdown of the triose phosphates, and they would presumably accumulate if niacin or nicotinamide were absent. As methyl glyoxal was observed to be increased in human tissue in vitamin deficiency states (329) there is again the possible implication that the methyl glyoxal arose from an accumulation of the trioses phosphates, under some effect of the redox state.

The redox state itself depends on the availability of electron acceptors capable of encouraging electron transfer reactions. When a biological system is metabolizing it produces an electric current (3). The coulombic output would be indicative of the net amount of electron transfer within the biological system. Diversion of the electrons from their normal pathway by any unspecific electron acceptor interrupts normal metabolism.

The ultimate electron acceptors in normal metabolism are oxygen atoms which seek electrons to complete their outer shells. That which becomes oxidized becomes a good acceptor because it becomes electron deficient. Although dihydroxyacetone or glyceraldehyde are electronically neutral they can be classified, in relation to electron acceptors, as good donors (306). Likewise ketoaldehydes with their two electropositive carbons close together, behave in reaction as acceptors. The dehydration of dihydroxyacetone to methyl glyoxal is equivalent to the change of an electron donor into an acceptor and is also a redox change: the first or second carbon atom of the molecule increases its oxidation state at the expense of the third. Thus again it is possible that the redox states, by affecting the donor-acceptor balance, could affect the production of ketoaldehydes.

The failure to give a reason for the enzymic production of methyl glyoxal during glycolysis resulted in the disbelief that it played any role at all (174). No attention was paid to the possibility of a non-enzymic production until Riddle and Lorenze (257) suggested that methyl glyoxal was produced from glyceraldehyde by the action of polyvalent anions. However they made no report of oxygen's accessibility to the reaction mixture. Some of the polyvalent anions were the phosphate anion, fructose-6-phosphate, fructose-1,6-diphosphate, and glucose-6-phosphate, and these might account for the appearance of methyl glyoxal in glycolysis. Its presence might have been why so many observations were made that phosphate inhibited respiration and glycolysis (181) (204) (334). Similar to producing methyl glyoxal from glyceraldehyde might be its production from dihydroxyacetone. Dihydroxyacetone (322) was reported to inhibit respiration in the presence of 100 mM phosphate.

Generally then, it can be concluded that ketoaldehydes, although in some instances are produced enzymically during metabolism, are quite possibly, often produced non-enzymically due to the redox state and the presence of catalysts. Different redox conditions and their effect on sulfhydryls and coenzymes, seem to correlate with either the cancerous state or the normal state. Therefore it may be possible to infer the involvement of ketoaldehyde production in the proper maintainance of control over cell division.

VII. Ketoaldehyde Reactions.

Once ketoaldehyde production is examined it follows that it is necessary to consider how ketoaldehydes react. The carbonyl group is reactive because it is polarized. The electron density is low on the carbon but high on the oxygen. Adjoining chemical groupings donate electrons to the carbon or withdraw them further, rendering the carbonyl with a variety of reactivities. The polymerization of aldehydes, as a measure of their reactivity, was seen to vary inversely with the electron density on the carbonyl C (327). Biological activity of the carbonyl in amides such as anilide, carbamates, dithiocarbamates, and urea derivatives, was shown to be due to the reduced electron density of the carbonyl C made by the electro-negative N group (243). Unsaturated groups in biologically active compounds were demonstrated to have an electron withdrawing effect and it was this which gave the compounds their activity (242). Nucleophilic N,S,O, seek the electron deficient regions readily; for instance α or β angelica lactone (137) (138) (139) reacts through its unsaturated bonds with thiols. Hydrazone formation is common with carbonyls and the carbonyl derivative of the thiosemicarbazide, semicarbazide, and guanyl hydrazide have shown anti-growth powers similar to the free aldehydes themselves as mentioned earlier, because they do not permanently block the carbonyl but dissociate or are displaced by other natural nucleophilic groups within the cells.

A transfer of the carbonyl reagent from its adduct to another carbonyl is possible, and conversely the carbonyl can be transferred from its carrier to another nucleophilic target. For instance it has been suggested that upon entry into the cell the adduct may be hydrolysed (286). How easily the hydrolysis occurs would be a reflection of the polarity of

the parent carbonyl function. More probably the adduct in the cell reacts with a more reactive nucleophilic group and is displaced (286). As mentioned above the carbonyl can have varying reactivity depending on the neighbouring groups, and as the nucleophilic centers would have diverse reactivities depending on their location among other influencing groups, some very subtle and specific reactions are possible when different ketoaldehydes and their adducts encounter different cellular groupings. It has been emphasized here that the ketoaldehydes react with sulfhydryl groups. Glyoxal produced radiosensitivity by blocking -SH groups (19). The thiol content of proteins combined with DNA, that is of histones (158), has not escaped notice (154) (173), but phenyl and methyl glyoxals were recently shown to inhibit the acetylation of histones in vitro (4) (248). This inhibition could be due to thiol blocking or possibly to a reaction of the dicarbonyl with basic amino acids like arginine in the histone. It has been shown that the arginine residue in Ribonuclease T_1 was modified by reaction with phenylglyoxal and glyoxal (313). The propensity of the dicarbonyl to react with basic amines made it a useful reagent for the detection of nucleotide sequences in single stranded regions of transfer RNA (200). Such reactions of glyoxal with nucleic acids, nucleotides and their component bases was studied by Nakaya et al. (225). In particular 1,2-dicarbonyls favour reaction with guanine residues (288) and the synthesis of N-2-alkylguanines was performed with glyoxal, methyl glyoxal and Kethoxal on guanine (287). Further guanosine and other nucleic acid derivatives reacted with glyoxal and ninhydrin (131). The adduct methyl glyoxal bis (guanylhydrazone) was studied by Sartorelli et al. and was found to complex with DNA and inhibit nucleic acid synthesis (271). Kethoxal bis (thiosemicarbazone) also inhibited DNA synthesis (49).

Since ketoaldehydes react with guanine it is possible that they affect the cell entering S phase. Recently it has been noticed that poly adenosine and poly guanosine exist covalently bound to messanger RNA in the polyribosome (158). These different species of mRNA function at different phases of the cell cycle: poly A mRNA functions during the G_2 phase and it is believed to have an influence on structural proteins while poly G mRNA functions in the S phase and is believed to relate to S phase proteins (36). A reagent predisposed for guanine residues could definitely prevent S phase functions if not the whole cycle. It thus appears that reactions of biological significance may occur with ketoaldehydes.

Ketoaldehydes have herein been described as very important substances in controlling the division of cells. Ketoaldehydes have been shown to be produced naturally by tissues, to react with nucleophilic groups and to be eliminated by GSH and the glyoxalase enzymes. They are possibly the growth arresting components of tissue extracts observed for over half a century. Their importance is unquestionable yet only the one, methyl glyoxal, has been definitely shown to be inhibitory and to be produced naturally <u>in vivo</u>. The retine of Szent-Gyorgyi was said to be more reactive than methyl glyoxal (304) and still awaits identification. As such compounds are reactive they are usually bound to other compounds and this makes them difficult to isolate or identify. The work reported in this thesis describes first the observation that growing cells secreted a growth inhibitor, that this inhibitor turned out to be a ketoaldehyde, and this, on further analysis was identified as hydroxyethyl glyoxal.

METHODS AND MATERIALS

1. Table of Abbreviations And Trade Names.

BHI - Brain Heart Infusion, a bacterial culture medium

CCM - Culture Conditioned Medium. Medium used for some time to culture cells, as opposed to fresh medium.

Eagle's MEM - Eagle's Minimal Essential Medium (GIBCO)

GIBCO - Grand Island Biological Company

HBSS - Hank's Balanced Salts Solution (GIBCO)

Kethoxal - 2-keto,3-ethoxy butyraldehyde, Reg. Trademark of Upjohn Pharmaceuticals Ltd.

Lab. Strain - A strain of a bacterial species isolated many years previously and perpetuated in a lyophilized state and by culture on laboratory medium.

LCDC - Laboratory Center for Disease Control, Dept. National Health and Welfare, Ottawa. Formerly the Canadian Laboratory of Hygiene.

Medium 199 - A cell culture medium (GIBCO)

NCDM - Neisseria Chemically Defined Medium Ref. 164.

PPLO - Pleuro-pneumonia-like organisms, a mycoplasma

Recent Isolates, Strains of bacteria isolated from source within the previous few months. Samples maintained lyophilized since first subculture.

Survival Number - Lowest number of bacteria in the inoculum, which survives inhibition. The lower the number the more resistant is the strain to inhibition, since the fewer the cells which survive, the more individually resistant they must be. See Exp. 5.

2. Animal Cells Used In This Study.

ANIMAL CELL	SOURCE
HeLa	American Type Culture Collection, Washington.
HeLa	From Dr. T.P. Brent, brought from Chester Beatty Res.Lab. London, England.
Human Amnion FL, Diploid	Baltimore Biological Labs.Div.of Bioquest
Human Embryonic Kidney, Diploid	"
Human Embryonic Lung, Diploid	"
Human Embryonic Intestine, Diploid	American Type Culture Collection, Washington.
Primary Human Amnion	Ottawa Civic Hospital via Virus Labs,LCDC
Human Kidney, Diploid (HK)	Virus Labs. LCDC, Ottawa.
Rhesus Monkey Kidney	"
African Green Monkey Kidney	"
L5178Y Mouse Lymphoblast	Dr. G. E. Fischer, Dept. Pharmacology, Yale Univ.

3. Growth of Monolayers of Mammalian Cells.

Medium 199 in dry powder form (Grand Island Biological Company, Grand Island New York, U.S.A.) without $NaHCO_3$ or Glutamine, was dissolved in double distilled water and dispensed into 250 ml volumes in 500 ml flasks with cotton stoppers. The medium was auotclaved 15 min. at 20 lbs. pressure and 120°C. When cool sterile solutions of glutamine and $NaHCO_3$ were added, the latter to bring the pH to 7.4.

For culturing the cells calf serum was added up to 10% of the volume. Cells were suspended in the medium with serum and 10 mls of the suspension were inoculated into 250 ml plastic culture bottles (Falcon Plastics, Becton-Dickenson Co. Ltd.) which were then held at 37°C. The used medium was poured off after an appropriate period of time, and was replaced by fresh medium. All handling of operations was done with asceptic technique.

4. Growth of L5178Y Mouse Lymphoblasts in Suspension.

Fischer's Medium in liquid, concentrated 10 x (GIBCO) was diluted with sterile double-distilled water, and calf serum was added to 10% by volume. Cells were suspended in the medium in a spinner flask at 37°C.

5. Calibration of the Coulter Counter.

L5178Y cells

Growing cultures of the L5178Y cell were checked by the microscope to attest to their health. Healthy cultures were lacking in debris and clumps and displayed only round cells of the character known for this strain. As these cells do not form monolayers but retain their individual lymphoblastic nature they are very well suited for spinner culture and Coulter Counter usage as clumping is not a problem.

L5178Y cells were collected and diluted into Hanks Balanced Salts

Solution. While the cells were made to pass through the 70 μ aperture tube the 1/AMP and the 1/APC switches were adjusted to bring the screen impulses into view so that the most intense band of pulse heights barely reached above the center of the screen. As the culture contained little other than individual L5178Y cells the pulse heights were taken to represent the cells. The distribution of the cell sizes were set, by the instrument switches to fit the center of the screen. The number of cells in each 'window' of four threshold units was counted to get a size distribution.

6. Counting L5178Y Cells.

Samples of cells were withdrawn from a spinner flask and diluted in particle-free HBSS at room temperature. The Coulter Counter Model B (Coulter Electronics Ltd., Front St., Toronto) was fitted with the 70 μ aperture tube and the settings were:

1/AMP	1
1/APC	2 (Aperture Current)
Gain Vernier	50
Matching Switch	64H
Lower threshold	10
Upper threshold	∞

7. Ragweed Pollen, 70 μ Aperture Tube.

Using ragweed pollen supplied by Coulter Electronics the following procedure was used to calibrate the mean threshold size in terms of cubic microns. One hundred miligrams of pollen were mixed with 95% alcohol in a small vial (1 ml). A drop of the suspension was put in about 10 ml of HBSS and was made to pass through the 70 μ aperture tube. Instrument settings were set to put the main intensity of pulse heights into the

center of the screen. A reasonable size distribution display was found with the settings at 1/AMP=4 and the 1/APC=2. The count was chosen at about 20,000 by diluting the solution with HBSS till this count was achieved when the lower threshold was set at 10 and the upper threshold was set at 100. The actual count was found at 21,748 on the average of four counts. It is known that the mean size of the ragweed pollen supplied was 3883 cu. μ. Therefore the threshold value of the mean of the size distribution would indicate 3883 cu. μ. Further counts were taken to by raising the lower threshold level until the counts were half of 21,748 which is 10,874. This number was found at a lower threshold level of 53, the mean particle threshold. Dividing 53 into 3883 gave the cu. μ per threshold setting. The quotient was 73.3 μ^3. To measure and count the L5178Y cell the setting 1/AMP must be at 1 while the 1/APC is at 2. By changing the 1/AMP setting from 4 to 2 the μ^3 per threshold setting are halved to 36.6. At a setting of 1/AMP equal to 1 the μ^3 per threshold setting are 18.3. The distribution of cell size for the L5178Y varies about a mean threshold of 55 which would give a mean size of 1006 μ^3 (55 x 18.3).

8. Growth of Bacteria.

All bacteria grown in this study were maintained by the Lab.Center for Control (LCDC) under the guidance of Dr. C. P. Kenny. Bacteria were cultured on Columbia Blood Agar (100), washed off and suspended in Neisseria Chemically Defined Medium (NCDM) (164). Where liquid cultures were needed organisms were grown in NCDM. All identifications, serotyping, etc., were performed by the LCDC.

9. Counting Bacteria.

Suspensions of bacteria in particle-free diluent (Hanks Balanced

Salts Solution, GIBCO) were counted on a Coulter Counter Model B fitted with a 35 μ aperture tube and settings:

1/AMP	$\frac{1}{4}$
1/A.C.	0.707
Gain	50
Match Switch	32 L
Lower Threshold	5
Upper Threshold	100

The background counts were negligible (30 to 150 counts per sample of bacteria counting 50,000 to 100,000). Counts were made on dilutions of a culture sample and the values recorded represent the average of three instrument counts.

10. Assay of Growth Inhibition of Bacteria.

Suspensions of the test organisms were made in NCDM after washing off Columbia Blood Agar (100). Samples of the suspension were counted on the Coulter Counter as described elsewhere. Suspensions were made ranging over 10^8 organisms in one ml and were carefully diluted to 1 x 10^8 organisms per ml. Separate dilutions by a factor of 10 were made of this one stock to provide concentrations of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 organisms per ml. One ml aliquots of these suspensions were inoculated into a series of sample tubes usually containing a mixture of fresh medium 199 and the inhibitory material to be tested. Growth in the tubes after 24 hrs was assessed on the basis of turbidity. This assessment could be performed with relative ease if the degrees of turbidity were only categorized into four. Complete growth, as in fresh medium only, was marked by very heavy turbidity, especially seen by shaking the tube and resuspending that which had settled out. The acidity accompanying this growth turned the medium

bright yellow from its action on the phenol red. However colour was not taken as the primary indication of growth, rather turbidity was. Complete growth was designated as 4+. Somewhat less sediment, observed to be present after resuspending by shaking, yet decidedly less turbid than the full growth in fresh medium, was designated as 3+. The colour of 3+ was often a warmer yellow yet retained an orange tinge. Still less turbidity was designated as 2+ growth. Here a small amount of sediment was re-suspended on shaking but turbidity was weak; colour was often a plain orange. Minimally detectable growth was designated 1+. Here turbidity was definitely visible but very slight, it contrasted to the clear non turbid appearance of fresh medium, colour was usually a reddish orange. In some cases doubt as to whether growth had existed was depicted as $\pm$. Non growth, or growth inhibition, designated as -, was seen by a clear liquid in the assay tube. On shaking, no turbidity could be produced. Occasionally specks or particles were seen, these due to clumped cells from the inoculum. In the early part of this work the content of these tubes was plated on agar to detect any viability in the organisms. No viability on agar was detected in any of those tubes previously judged as inhibited.

In short this distinction of only four growth levels provided a reliable and reproducible method as opposed to one more fine which would distinguish perhaps ten levels. In such it would be difficult to claim observing differences between levels. The bulk of these operations, that is using many duplicate tube assays in multiple series, precludes the use of a method for counting or measuring bacterial production in individual tubes. The purpose of these assays was to assess relative growth of similar inocula in different media or dissimilar inocula in one medium. Therefore the use of controls (bacteria in fresh medium), always gave the

maximum growth possible, which is taken as 4+. Any growths less than this are classed in relative order and assigned easily to +, 2+, or 3+. Thus the method can be justified as useful and accurate within the limits of its pretensions.

11. Sephadex Column Chromatography.

(a) Column Preparation

In this study glass columns measuring 2.8 x 40 cm were used. Sephadex grades 50, 25, 15, and 10 were employed. Each grade was washed by suspending in distilled water, and by pouring off the water after the dextran had settled. This operation was carried out a minimum of four times to remove fines. A slurry of suspended Sephadex was poured into a column of distilled water through an extension attached to the top. As the Sephadex settled into the column the water moved to the extension from where it was removed. The extension was removed when the Sephadex would settle no further. A reservoir bottle was attached by a tube to the top plug of the column and water was run through the column to stabilize the uniformity of compaction of the Sephadex. If shortening of the bed occured more Sephadex slurry was stirred into the top of the bed and allowed to settle. One quarter inch of space was left between the top plug and the upper surface of the bed.

(b) Void Volume Measurement

To measure void volumes of the columns a solute of weight greater than the exclusion size had to be chosen. To be excluded by the largest pore size of the Dextran grain used (Sephadex G 50) a solute of weight greater than 50,000 was needed. Human Serum Albumin (MW 70,000) was used dissolved in distilled water to give a 10% solution. One tenth of an ml of this solution was pipetted onto the top of the bed. After it seeped

in, a little water was added to keep the top of the bed wet. More water was added at about one tenth of an ml at a time till the albumin was about one half inch into the bed. Then the column was filled and the reservoir was attached. Collecting fractions began the moment the albumin was added. Fractions of four ml were collected and their O.D. was read at 280 nm void volumes.

(c) Void Volumes

With the Sephadex G 50 column absorbance first appeared in the contents of tube 8, peaked at tube 10, and was again low in tube 12. With the Sephadex G 25 column absorbance appeared in tube 7, peaked at tube 10 and was nearly absent again in tube 12. With the Sephadex G 15 column absorbance first appeared in tube 5, peaked at tube 7, and was nearly absent by tube 9. With the Sephadex G 10 column absorbance first appeared in tube 3, peaked at tube 5 and was nearly absent in tube 8.

(d) Molecular Weights

Sephadex G 50 excludes molecules ,in the void volume, over a weight of about 50,000. Sephadex G 25 excludes, in the void volume, molecules over a weight of about 25,000. The weights excluded by G 15 and G 10 are about 15,000 and 700 respectively. This information is given by Pharmacia, the manufacturers of Sephadex.

12. Bacterial Strains Used In This Study.

SPECIES	LCDC #	OTHER DESIGNATIONS			VIRULENCE
E. coli	138	Serotype	B		non V
"	235	Serotype	O 119		V
"	317	"	O 55		V
"	324	"	O 26		V
"	540	"	O 127		V
"	549	"	O 14		non V
"	598	"	O 26		V
"	673	"	O 14		non V
"	760	"	O 26		V
"	765	"	O 126		V
S. typhi					V
N. gonorrhoeae		Colony type	I		V
			II		V
			III		non V
			IV		non V
N. meningitidis					
	158	Serotype	D	Lab. Strain	non V
	1027	"	A	"	non V
	1628	"	C	"	non V
	2092	"	B	"	non V
		"	Slaterus X	"	non V
		"	Slaterus Y	"	non V
		"	Slaterus Z	"	non V
	604	"	A	Recent Isolate	V
	552	"	B	"	V
	13	"	C	"	V
	247	"	X	"	V
	553	"	Y	"	V
	564	"	Z	"	V
S. aureus	2919	Phage Group	I		V
	2695	et al.	II		non V
			III		V
			IV		non V
			Miscellaneous		non V

RESULTS

"Poets virtually transcend their finite selves when ready for the creative act. Schiller, it is reported, had an olfactory delusion (which arises in the limbic system), for he could smell rotten apples when he sat down to write". *

Erwin Di Cyan

Perspectives In Biology and Medicine

Vol. 14, p 639 (1971)

* See Experiment 20.

TABLE 1. Growth of E. coli strain 014 (+ indicates growth) after 24 hrs

MEDIUM	Number of organisms inoculated	10^8	10^7	10^6	10^5	10^4	10^3	10^2
	2 ml used medium	+	+	+	-	-	-	-
	1 ml fresh medium and 1 ml used medium	+	+	±	-	-	-	-
	2 ml fresh medium	4+	4+	4+	4+	4+	4+	4+

A. BACTERIAL GROWTH INHIBITION - EXPERIMENTS 1 to 7.

(1) Growth Inhibition of E. coli 014

Purpose:

To assay the antibacterial activity of used tissue culture medium.

Experimental:

E. coli organisms were counted by the Coulter Counter and inoculated into fresh medium and medium used by HeLa cells for a couple of days. Growth in the tubes of medium 199 and serum was observed after 24 hours and was indicated by degrees of turbidity on a scale of four degrees marked as +, 2+, 3+, 4+, the latter denoting complete growth within a medium turned yellow by the effect of acid on phenol red. Colour of these growth tubes was helpful in indicating the degree of growth but turbidity was taken as the main measure of abundance. Absence of growth is indicated by a dash: -, and doubt between growth and its absence is indicated by $\pm$. Added fresh medium could overcome lost nutrients.

Results: TABLE 1.

The heavier the inoculum the more growth was visible but HeLa cell medium seemed to inhibit the growth of even the heavy inoculum 10^8, 10^7, or 10^6 bacteria, in comparison to the growth of the controls. The inhibition was complete with inocula of 10^5 organisms or less, regardless of nutrients.

Conclusion:

Growth of E. coli 014 can be inhibited by HeLa cell conditioned medium. The used medium is not depleted of nutrients as added nutrients did not change the growth pattern in it.

TABLE 2. Growth in 1 ml of 199 and 1 ml of conditioned medium from HeLa cultures. (All strains grew in the control, 2 ml of fresh medium.

No. of organisms		10^8	10^7	10^6	10^5	10^4	10^3	10^2
CCDC no.	Serotype							
598	O 26	4+	4+	4+	2+	-	-	-
765	O 126	4+	4+	3+	2+	+	-	-
760	O 26	4+	4+	3+	2+	+/-	+/-	-
540	O 127	4+	4+	2+	+	-	-	-
324	O 26	4+	4+	4+	2+	-	-	-
317	O 55	4+	4+	3+	2+	+/-	-	-
235	O 119	4+	+	+	+	-	-	-
673	O 14	2+	+	+/-	-	-	-	-
549	O 14	2+	+	+/-	-	-	-	-
138	B	+	+	+	-	-	-	-

(2) Other E. coli Strains

Purpose:

To observe the growth inhibition of a variety of E. coli strains to ascertain the general extent of the inhibition.

Experimental:

A number of strains of E. coli isolated from diverse sources were tested for growth inhibition by the HeLa cell conditioned medium. Most were strains classified as virulent by the Communicable Disease Center for their involvement in infantile diarrhea (103).

Results: TABLE 3.

Serotype 014 and B, classed as non-virulent by CDC (103) were inhibited in CCM. Heavy inocula (10^8, 10^7, and 10^6 organisms) grew somewhat but much less than controls: 2+ or + as compared with 4+. On the other hand strains classed as virulent by their implication in diarrheal disease survived inhibition in such low numbers as 10^5 and 10^4 in the inocula. As a group they appeared to be more resistant to inhibition.

Conclusion:

Since strains of serotype O 26, O 55, O 119, O 126, and O 127 are reported to occur frequently in diarrheal disease they are considered more virulent than the strains of serotype O 14 or B, (103). In this study the virulent strains appeared to be more resistant to growth inhibition by a factor in CCM than the other strains, therefore the type of inhibition seen here may only apply to non virulent strains.

TABLE 3. Growth of 10^5 organisms placed in 1 ml medium 199 and 1 ml medium conditioned by HeLa cells.

Organism		No. of Strains Tested	Growth
Staphylococcus aureus			
Phage group	I	3	Yes
	II	8	No
	III	3	Yes
	IV	1	No
	Miscellaneous	3	No
Neisseria meningitidis			
Serotype	A Lab. Strains	2	No
	B "	2	No
	C "	2	No
158	D "	1	No
Neisseria gonorrhoeae			
Colony Type	I	4	Yes
	III	5	No
Escherichia coli			
Serotype	014	3	No
"	026	3	Yes
Salmonella typhi		4	Yes

(3) Survey of a Variety of Bacteria

Purpose:

To survey other bacteria of varying degrees of virulence.

Experimental:

A variety of bacterial genera were tested for growth inhibition. Strains were distinguished by the degree of virulence according to existing standards.

Results: TABLE 3.

At an inoculum of 10^5 organisms growth was discovered after 24 hours in CCM, of all strains of S. aureus belonging to Phage group I or III, of all N. gonorrhoeae strains of Colony Type I, of all E. coli serotypes 026, and of all Salmonella typhi. Inhibition of growth was evident with S. aureus Phage groups II, IV and Miscellaneous, with all strains of N. meningitidis available, with N. gonorrhoeae Colony Type III and E. coli serotype 014.

Conclusions:

An interesting aspect of the inhibitor from HeLa cells seems to be that it inhibits primarily the lesser virulent organisms in a number of bacterial genera, while the virulent strains are not so affected under the conditions of concentration versus the number of organisms. See discussion for explanation of virulence criteria for the strains used.

(4) Mouse Challenge Test for Meningococcus Virulence

Purpose:

To compare the infectivity in mice of N. meningitidis strains from human patients and carriers, and strains perpetuated in laboratory culture.

Rationale:

Of those bacteria used in this study (Exp. 3) the degree of virulence, known by their disease producing capability, had been estimated by virtue of a previously established relation to some other characteristic of the organism. For example virulence of S. aureus had been correlated to its phage type group (88) (214). Virulence of N. gonorrhea had been classified by its colony type (160)(161) (162), E. coli virulence had been correlated with certain serotypes (103). For N. meningitidis a test was needed which could distinguish strains freshly isolated from patients and carriers, from strains perpetuated on laboratory medium. Such a test had been devised (163) and this procedure was adopted in this experiment.

Experimental:

The mouse challenge test used black mice (C 57) purchased from Flow Labs Inc. inoculated with organisms in chemically defined liquid medium and 2.5% gastric mucin. Sex of mice made no difference to the results but each lot tested was all of one sex. The mice were not older than 2 months and weighed between 15 and 18 gm.

The organisms were grown on blood agar to 18 hrs then were washed off the agar with NCDM (164) at 37°C. The number of organisms in the suspension was counted by the Coulter Counter and diluted to 2×10^8, 2×10^7, 2×10^6 down to 2×10^1 per ml. One half ml of these

TABLE 4. Deathof mice infected with laboratory strains of N.meningitidis

	NUMBER OF DEAD MICE OUT OF TEN GIVEN LAB. STRAINS						
Inoculum	1027A	2092B	1628C	158D	Slat.X	Slat.Y	Slat.Z
$1x10^8$	8	9	10	8	10	10	9
$1x10^7$	9	9	6	7	9	9	9
$1x10^6$	4	2	1	4	6	9	6
$1x10^5$	0	0	1	2	3	4	4
$1x10^4$	1	0	0	1	1	3	2
$1x10^3$	0	0	0	0	0	1	1
$1x10^2$	0	0	0	0	0	0	0
$1x10^1$	0	0	0	0	0	0	0
Control	0	0	0	0	1	0	0

TABLE 5. Death of mice infected with recently isolated strains of *N. meningitidis*.

	NUMBER OF DEAD MICE OUT OF TEN GIVEN DISEASE STRAINS						
Inoculum	A 129	604A	552B	13C	247X	553Y	564Z
$1x10^8$	7	10	10	10	10	10	9
$1x10^7$	8	10	10	10	10	10	8
$1x10^6$	10	10	10	10	10	10	7
$1x10^5$	10	8	8	9	10	10	7
$1x10^4$	5	7	9	9	9	10	5
$1x10^3$	0	6	9	8	9	8	3
$1x10^2$	2	4	6	9	8	10	1
$1x10^1$	0	2	3	4	5	9	0
Control	0	0	0	0	0	0	0

suspensions was mixed with 0.5 ml of 5% gastric mucin in saline at 37°C. Mice were injected intraperitoneally within one half hour of washing the organism off blood agar. A loopful of the suspension was plated on blood agar at this time to verify the organism's viability.

The control mice were inoculated with NCDM and gastric mucin at 37°C. Ten mice were put in each group and death of the challenged animals was noted at 72 hrs after inoculation. The LD_{50} is the minimum number of bacteria necessary to kill at least 50% of the population, or when 5 of the 10 mice of each group die.

N. meningitidis is serotyped into at least 7 groups: A,B,C,D,X,Y.Z. Of these strain 1027 A, 2092 B, 1628 C, 158 D, Slaterus X, Slaterus Y, and Slaterus Z were originally isolated years ago from spinal fluid of cases of meningococcal infection. Since isolation they have been grown on laboratory medium. Strain 604 A, 552 B, 13 C, 247 X, 553 Y, and 564 Z, have all been recently isolated from cases in Canadian institutions, (D has never been found in more than 2 cases on record). The recently isolated strains have been set aside in vials by lyophilization immediately after their acquisition and therefore are always available as close to their condition at isolation as possible. Strain A 129 was received years ago yet samples were kept lyophilized in their condition after a few passages on laboratory medium.

Results:

Table 4 indicates the LD_{50} of N. meningitidis strains perpetuated on laboratory medium for a number of years. The LD_{50}'s are from 10^7 to 10^6 organisms. For strains recently isolated from diseases of patients the LD_{50}'s (Table 5) were 10^2 to 10^1 organisms. One laboratory strain A 129 gave an LD_{50} of 10^4 but it had been kept lyophilized a long time with a minimum number of Laboratory passages.

Conclusions:

The strains of N. meningitidis used here can be classified as more virulent (LD_{50} 10^2 - 10^1) pathogens, and less virulent (LD_{50} 10^7 - 10^6) pathogens. This demarcation coincides with whether the organisms were recent isolates from humans or were laboratory strains. With a measurable difference in the degree of virulence thus ascertained these strains of N. meningitidis can now be examined for their ability to survive in the presence of the inhibitory factor from human cells.

TABLE 6. Growth (+) of inoculum of Organisms after 24 hrs
(All strains grew in the control fresh medium)

Strain	No. of organisms in inoculum					
	10^7	10^6	10^5	10^4	10^3	10^2
1027 A	2+	+	-	-	-	-
	2+	±	-	-	-	-
129 A	4+	3+	3+	2+	-	-
	4+	3+	3+	2+	-	-
604 A	4+	4+	4+	2+	±	-
	4+	4+	4+	2+	+	-
2092 B	2+	2+	+	-	-	-
	2+	2+	+	-	-	-
552 B	4+	4+	4+	4+	3+	+
	4+	4+	4+	3+	3+	+
1628 C	3+	2+	2+	-	-	-
	3+	2+	+	-	-	-
13 C	4+	4+	4+	2+	+	-
	4+	4+	4+	2+	+	-
158 D	2+	2+	2+	-	-	-
	2+	2+	2+	-	-	-
Slat X	4+	3+	3+	2+	-	-
	4+	3+	3+	+	-	-
247 X	4+	4+	4+	3+	3+	+
	4+	4+	4+	4+	3+	+
Slat Y	4+	3+	3+	2+	-	-
	4+	4+	3+	2+	-	-
553 Y	4+	4+	4+	3+	2+	+
	4+	4+	4+	3+	2+	+
Slat Z	4+	3+	3+	2+	-	-
	4+	4+	3+	2+	-	-
564 Z	4+	4+	4+	4+	3+	-
	4+	4+	4+	4+	3+	-
* 551	4+	4+	4+	4+	4+	+
	4+	4+	4+	4+	4+	+

* Untypable strain isolated from an 18 year old Ottawa girl who died a few hours after admission to the Civic Hospital.

(5) Degrees of Virulence and Survival in CCM

Purpose:

To measure the survival of virulent and less virulent strains of N. meningitidis in the presence of conditioned medium.

Experimental:

Strains of N. meningitidis were grown as in experiment 4 and bacteria were diluted and counted in NCDM. In duplicate tubes one ml of the counted suspension was added to one ml of conditioned HeLa cell medium and growth was observed up to 72 hrs.

Results: See TABLE 6.

Conclusion:

In every case a virulent strain, as defined by its isolation from humans and by its LD_{50} in the mouse challenge test, was compared to less virulent strains of the same serotype perpetuated on laboratory medium. Serotype seemed to make little difference to the degree of virulence of the strains measured as virulent in Exp. 4. The latter are more resistant to growth inhibition by CCM than are the laboratory strains in every case.

The lowest number in the inoculum capable of surviving the inhibition could indicate how individually resistant the organisms are. Low numbers could imply greater resistance than large numbers. The CCM Survival Number could thus be defined as a measure of resistance and possibly as a measure of virulence.

These results, in addition to those with other genera, would tend to confirm that regardless of the bacterial species used in these studies the degree of virulence is capable of being measured as the degree of resistance to inhibition by the factor from HeLa cells.

TABLE 7. Growth of S. aureus group II (strain 2695) in CCM, in CCM used by strain 2695 for 8 hrs, and CCM used by S. aureus group I (strain 2919)

Medium	Number of Group II Strain Inoculated					
	10^7	10^6	10^5	10^4	10^3	10^2
CCM control	4+	+	-	-	-	-
	4+	+	-	-	-	-
CCM used by 2695 (strain of group II)	4+	4+	2+	2+	±	-
	4+	4+	+	2+	-	-
CCM used by 2919 (strain of group I)	4+	3+	2+	±	-	-
	4+	4+	+	+	-	-

(6) Resistance of Staph. Aureus Groups I and II

Purpose:

To investigate whether the virulent strains of bacteria are more resistant to the inhibitor by actively destroying it.

Experimental:

Exp. 3 demonstrated that S. aureus group I resisted the inhibition suffered by group II. Whether this resistance takes the form of destruction of the inhibitor, or blocking of the inhibitor, or some other process, is unknown but a measure of the inhibitor remaining after exposure to group I and II strains is desirable.

The quantitative method of assaying the inhibitor's strength in used culture medium is to observe the survival of a series of bacterial dilutions in equal volumes of the medium. A series of bacterial dilutions was therefore inoculated into normal CCM and into CCM which had previously supported a large inoculum (10^7 organisms) of a strain of S. aureus for 8 hrs at 37°C and which had been filtered through a membrane of 0.20 μ pore size. Samples of the filtered medium were plated to test for sterility before the serial dilutions of the bacteria were inoculated into duplicate tubes of the filtrate.

Results: Table 7.

The survival of organisms from inocula of 10^5 or less indicated, in comparison to the control, that less inhibitory activity was present in the CCM. The higher survival of organisms in CCM used by strain 2695 than by 2919 may indicate a difference in the amount of inhibitor remaining.

Conclusion:

Large numbers (10^7) of organisms of S. aureus strains 2695 and 2919 can

destroy the inhibitor perhaps enzymically or modify its ability to inhibit, as shown by a cut off point of 10^3 test organisms compared to 10^5 in the control. Both strains demonstrated resistance by lowering the inhibitor level, but the virulent strain, although it is known to be more resistant to growth inhibition, is not more destructive of inhibitor (1+ growth is more inhibited than 2+ for 10^4 organisms). The greater resistance then, of virulent strains, may not be a greater power to destroy the inhibitor but perhaps to a greater degree than less virulent strains they can minimize contact of the inhibitor with the cell division mechanism to avoid inhibition, and with the destructive capacity of the cell.

destroy the inhibitor perhaps enzymically or modify its ability to inhibit, as shown by a cut off point of 10^3 test organisms compared to 10^5 in the control. Both strains demonstrated resistance by lowering the inhibitor level, but the virulent strain, although it is known to be more resistant to growth inhibition, is not more destructive of inhibitor (1+ growth is more inhibited than 2+ for 10^4 organisms). The greater resistance then, of virulent strains, may not be a greater power to destroy the inhibitor but perhaps to a greater degree than less virulent strains they can minimize contact of the inhibitor with the cell division mechanism to avoid inhibition, and with the destructive capacity of the cell.

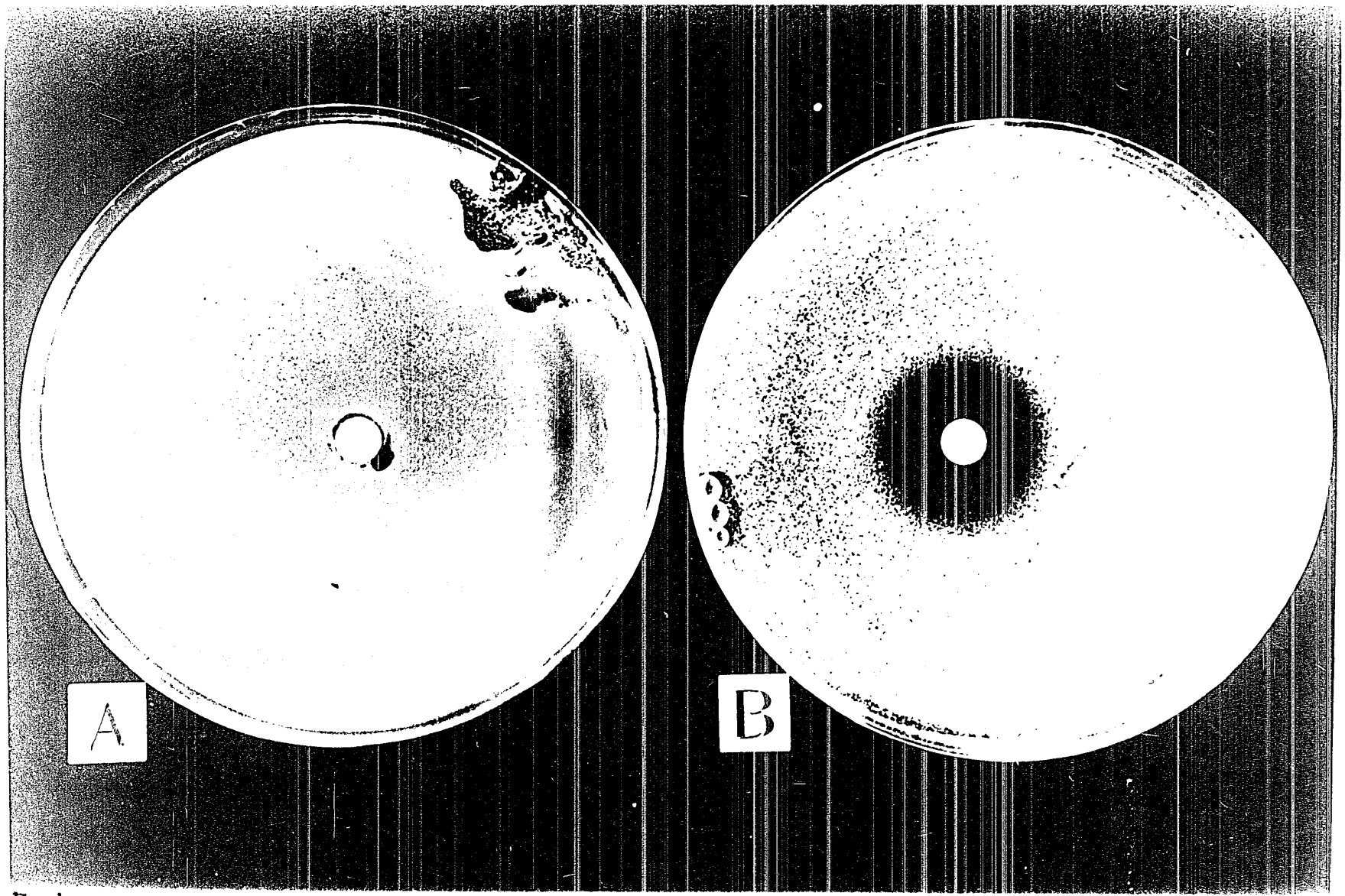


Fig 1.

(7) Disk Assays

Purpose:

To observe inhibition by disk assays on seeded agar plates.

Experimental:

The following tests were done using a strain of Staphylococcus aureus from phage group I as an example of a virulent strain, and a strain from phage group II as the less virulent strain. Sterile filter paper disks (diameter 5 mm) were soaked in conditioned culture medium (CCM) from HeLa cells, which had been concentrated by partial lyophilization to about one tenth its volume. The disks, when dry, were placed on agar plates seeded with equal numbers of the strains to be tested.

Results:

The photograph Fig. 1 shows the inhibition of a group II strain (B), but not of a group I strain (A).

Conclusion:

Disk assays show the same type of inhibition as do tube assays.

B. PRODUCTION OF THE INHIBITOR*- EXPERIMENTS 8 - 12.

(8) Yield of the Inhibitor*

Purpose:

To measure the yield of the inhibitor* by HeLa cells.

Experimental:

Plates of BHI agar were seeded with E. coli 014 and sterile wells(d:10 mm) placed on the surface. Into the wells 0.2 ml aliquots of conditioned HeLa cells media were poured, or fresh medium 199 as a control. There were duplicate plates for each sample of medium, five wells on each plate, and the medium had fed cultures either for one day, two days, or three days. After overnight growth of the bacterial lawn the zones of inhibition were marked and the transverse diameter of each zone was measured. Due to a washing pressure of the high level of the solution in the wells there was a zone of no growth even around the control, the area of this zone was taken as the zero value of inhibition.

Results: See TABLE 8.

The average area in mm^2 was 96 for fresh medium, 137 for medium conditioned 1 day, 213 for medium conditioned 2 days, and 310 for medium conditioned 3 days: Fig. 2 .

Conclusion:

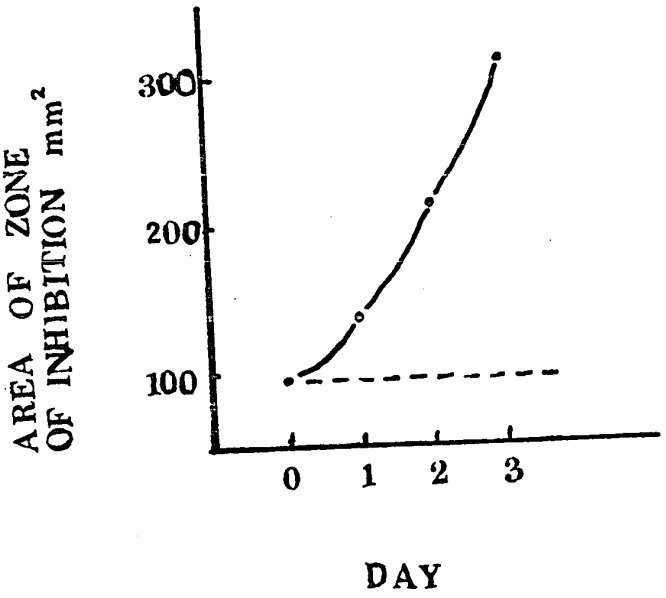
The average area of growth inhibition is seen to increase with medium of increasing exposure to growing HeLa cells, suggesting the accumulation of an inhibitor in the medium with time, perhaps varying directly with the growth of the cells.

*Possibly several inhibitors may be involved but one type of activity, later it is pointed out that only one substance was isolated. No evidence is available however that only one inhibitor exists.

TABLE 8. Sizes of Zones of Growth Inhibition

Medium 199	SIZE OF ZONE diameter in mm	r (mm)	r^2 (mm^2)	$r^2 \times \pi$	AREA AVERAGE
Fresh	11	5.5	30.25	95	
	11	5.5	30.25	95	
	10	5	25	79	
	11	5.5	30.25	95	
	10	5	25	79	
	12	6	36	113	
	12	6	36	113	
	12	6	36	113	
	11	5.5	30.25	95	
	10	5	25	79	
					96 mm^2
Conditioned 1 day	14	7	49	154	
	11	5.5	30.25	95	
	15	7.5	56.25	177	
	15	7.5	56.25	177	
	14	7	49	154	
	10	5	25	79	
	13	6.5	42	133	
	13	6.5	42	133	
					137 mm^2
Conditioned 2 days	18	9	81	254	
	18	9	81	254	
	18	9	81	254	
	17	8.5	72	227	
	15	7.5	56	177	
	14	7	49	154	
	16	8	64	201	
	17	8.5	72	227	
	17	8.5	72	227	
	14	7	49	154	
					213 mm^2
Conditioned 3 days	20	10	100	314	
	22	11	121	380	
	21	10.5	110	346	
	18	9	81	254	
	19	9.5	90	283	
	20	10	100	314	
	15	7.5	56	177	
	23	11.5	132	415	
	16	8	64	201	
	23	11.5	132	415	
					310 mm^2

Fig. 2 Amount of Inhibitor in Medium used by HeLa Cells



(9) Production in Different Media

Purpose:

To examine whether the inhibitor is produced by HeLa cells in medium other than medium 199.

Experimental:

HeLa cells were grown in medium 199, in Brain Heart Infusion Broth with Yeast RNA (165), and in Eagle's Minimal Essential Medium. Used medium was tested for inhibitory powers.

Results: TABLE 9

Growth of *S. aureus* strain 2695 (group II) after 24 hrs

	Fresh Medium	Used Medium
medium 199	4+	-
HIB/RNA	4+	-
Eagle MEM	4+	-

Conclusion:

HeLa cells secrete the inhibitor in any of these three media.

TABLE 10. Growth of S. aureus in medium 199 conditioned by various cell lines.

CELL LINE	STAPHYLOCOCCAL GROUP	
	I (2919)	II (2695)
HeLa (Ottawa)	4+	-
HeLa (London England)	4+	-
Human Amnion FL	4+	-
Human Embryonic Kidney	4+	-
Human Embryonic Lung	4+	-
Human Embryonic Intestine	4+	-
Human Kidney	4+	-
Rhesus Monkey Kidney	4+	2+
African Green Monkey Kidney	4+	4+
HeLa infected with PPLO	4+	4+
Human Amnion FL with PPLO	4+	4+

(10) Production by Different Cells

Purpose:

To observe if the inhibitory activity is produced by cells other than HeLa cells in medium 199.

Experimental:

A variety of cell lines were grown and used medium from each type was assayed for the inhibitory activity. 10^4 organisms of S. aureus 2919 and 2695 were inoculated in to the test medium. Some of the cell lines had acquired a detectable PPLO contamination.

Results: TABLE 10.

Growth of S. aureus strain 2695 was inhibited by medium conditioned by HeLa cells, Human Amnion FL cells, Primary Human Amnion Cells, Human Embryonic Kidney, Lung and Intestine cells, and Human Adult Kidney cells. Inhibition was not found in medium from Rhesus Monkey Kidney cells, African Green Monkey cells, HeLa cells or Human Amnion FL cells infected with PPLO.

Conclusion:

The human cells seem to make the inhibitor, but not the monkey cells or PPLO infected cells.

TABLE 11. Growth of S. aureus strains 2919 and 2695 in medium treated by changing the pH

pH of Treated Medium	Strain 2695	Strain 2919
3	-	4+
4	-	4+
5	-	4+
6	-	4+
7	-	4+
8	-	4+
9	-	4+
10	-	4+
11	-	4+
Fresh Medium	4+	4+

(11) Stability of The Inhibitor (i)

Purpose:

To study the stability of the inhibitor before attempting to purify it. (i): Effect of Hydrogen ion concentration on the inhibitory activity.

Experimental:

Aliquots of CCM from HeLa cells were adjusted to various hydrogen ion concentrations from pH 3 to pH 11 with HCl or NaOH (1 N) and were kept at room temperature for 24 hrs. Before testing each aliquot for inhibitor activity the samples were neutralized with appropriate quantities of sterile NaOH or HCl and mixed 1:1 with fresh medium 199. Tubes were inoculated with 10^4 bacteria and observed for growth. Strain 2919 and Strain 2695 are classed in Phage Groups I and II respectively.

Results: TABLE 11.

CCM retained its inhibitory activity whether held for some time at pH 3 or pH 11.

Conclusion:

pH changes from 3 to 11 do not eliminate the inhibitory activity of conditioned medium. Therefore it is fairly safe to purify the factor within these limits of pH if necessary.

TABLE 12. Growth response of S. aureus in CCM treated to different temperatures

Temperature of Treated Medium	Growth of 10^4 of Staph.Group I (2919)	II (2695)
-20° C	4+	-
0° C	4+	-
4° C	4+	-
24° C	4+	-
37° C	4+	-
45° C	4+	-
54° C	4+	-
80° C	4+	-
100° C	4+	-
121° C	4+	-
Fresh Medium	4+	4+

(12) Stability of The Inhibitor (ii)

Purpose:

To study the stability of the inhibitor (ii): with respect to temperature changes.

Experimental:

Samples of HeLa conditioned medium were maintained at temperatures ranging from -20°C to 100°C for 24 hrs before assay at 37°C when they were mixed 1:1 with fresh medium. Some samples were also autoclaved at 121°C for 15 minutes and were filtered, the filtrates were mixed with fresh medium for assay.

Results: TABLE 12.

CCM retained its inhibitory activity of Strain 2695 whether held for some time at -20°C or at 121°C.

Conclusion:

Temperature changes from -20°C to 121°C do not eliminate the inhibitory activity of used medium suggesting perhaps that the factor is heat stable.

TABLE 13. Growth response of E. coli 014 to CCM and to dialysed CCM

No. of Organisms	GROWTH IN fresh 199	CCM	Dialysis bag contents	Dialysate
10^6	4+	-	4+	4+
10^5	4+	-	4+	4+
10^4	4+	-	3+	3+
10^3	4+	-	+	+
10^2	4+	-	-	-

C. ISOLATION OF THE INHIBITORY ACTIVITY-EXPERIMENTS 13 to 17

(13) Dialysis of Used HeLa Cell Medium

Purpose:

To observe the behaviour of the inhibitor upon dialysis of the used medium.

Experimental:

With asceptic technique sterile dialysis bags (50 ml) were filled with HeLa conditioned medium and were dialysed against 250 ml sterile fresh medium under agitation at 4°C for 5 days. Bag contents and dialysates were assayed for inhibitory activity against E. coli 014. Growth of bacteria in 199 and used medium was compared with the dialysed preparations.

Results: TABLE 13.

Dialysis bag contents appeared to have the same inhibitory activity as the dialysate, less than the whole CCM but more than fresh medium.

Conclusion:

These results would suggest that the inhibitor is dialysable since inhibitory activity seemed to be diluted and distributed through both dialysate and dialysed material. The dialysis tubing used was capable of passing substances below the molecular weight of 30,000; therefore Isolation of the inhibitor should begin by separating material over this weight from material under this weight.

(14) Sephadex G 50

Purpose:

To isolate the factor using Sephadex G50 chromatography.

Experimental:

50 ml of CCM was frozen dried after collecting, pooling and filtering for sterility. The dry material was redissolved in water to 2 ml and was passed over a column (2.8 x 40 cm) of Sephadex G50 in water, and fractions of four ml were collected. Three peaks were obtained by determining the optical densities of each fraction at 280 nm (Fig. 3). The tubes of eluate comprising peak I (7-15), were pooled, as were tubes 16-25, (peak II), and tubes 26-42 (peak III). The pools were concentrated by freeze drying and reconstituted to 2.0 ml with water for further purification or with fresh medium for assaying. Fresh medium was also lyophilized and fractionated on a G50 column to provide a control for the assay. In this case the third peak contains the phenol red, and a control was needed to be certain it was not interfering with growth of the test organism. Growth of 10^4 organisms was noted in the fractions.

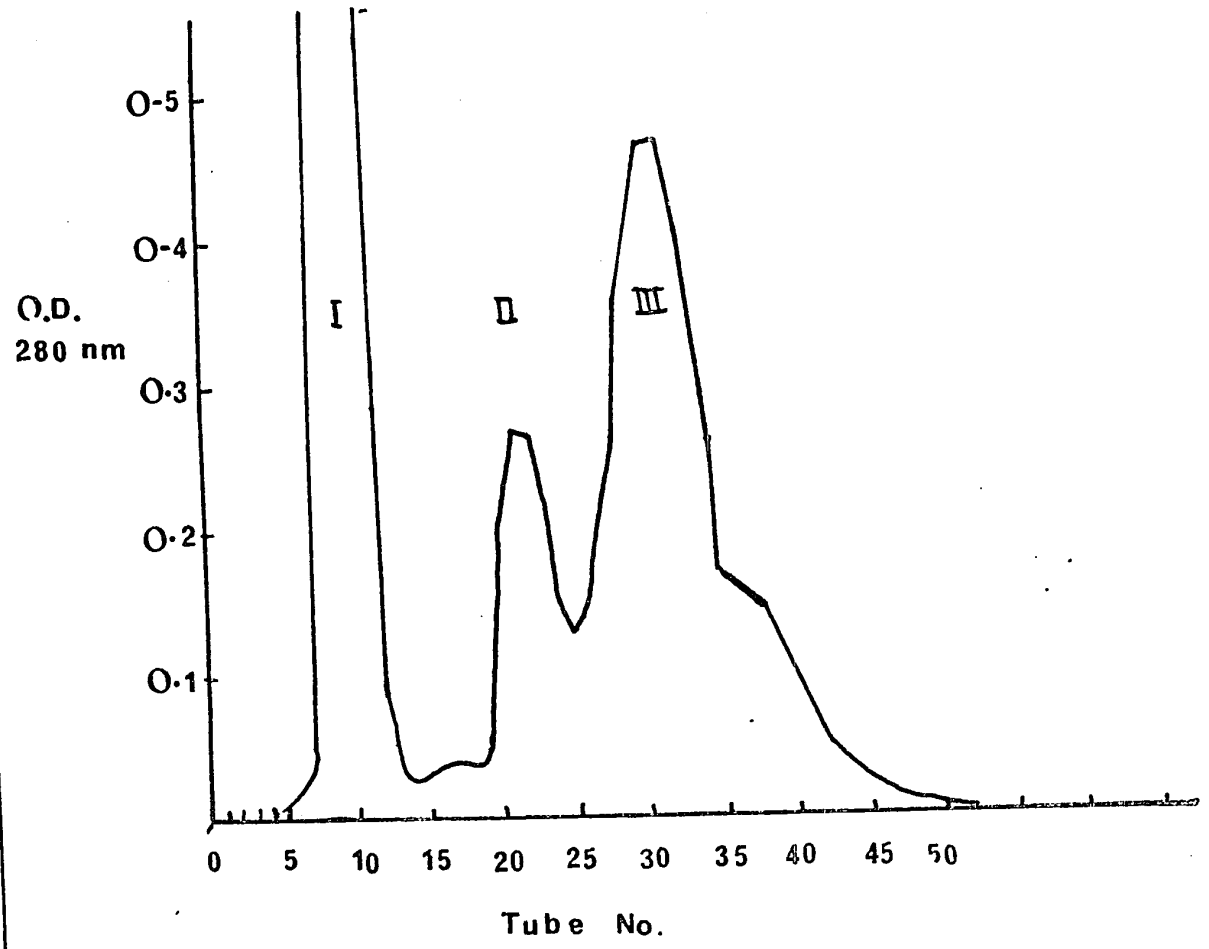
Results: Fig. 3 and Table 14.

Growth of Staphylococcus aureus strains from phage group I (2919) and II (2695) was observed in the presence of column fractions of HeLa cell conditioned medium except in peak number three. All peaks of fresh medium supported growth.

Conclusion:

The third peak of the G50 column fractionation contained the antibacterial activity.

Fig. 3 Absorbance of fractions from Sephadex G50



(15) Sephadex G 25

Purpose:

To isolate the factor using Sephadex G25 chromatography.

Experimental:

The third G50 peak of the conditioned medium, which contained the inhibitory activity, was concentrated by lyophilization, passed through a column of Sephadex G25, and fractions of 4.0 ml were collected. Two principle peaks were eluted with water. Void volume had been determined for the column as up to tube 12. Peak One was taken in tubes 14 to 21, peak Two was taken in tubes 22 to 30 (Fig. 4). The peak materials were concentrated to 1 ml by freeze drying. They were assayed for inhibitory activity; active samples were further purified.

Results: TABLE 15.

Test organisms were inhibited by material from the first G25 peak (Fig.4).

Conclusion:

The activity resided in the first peak, appearing after the void volume. The weak growth of even the virulent strain in the presence of these fractions is due to the effect of concentrating the inhibitory factor by freeze-drying.

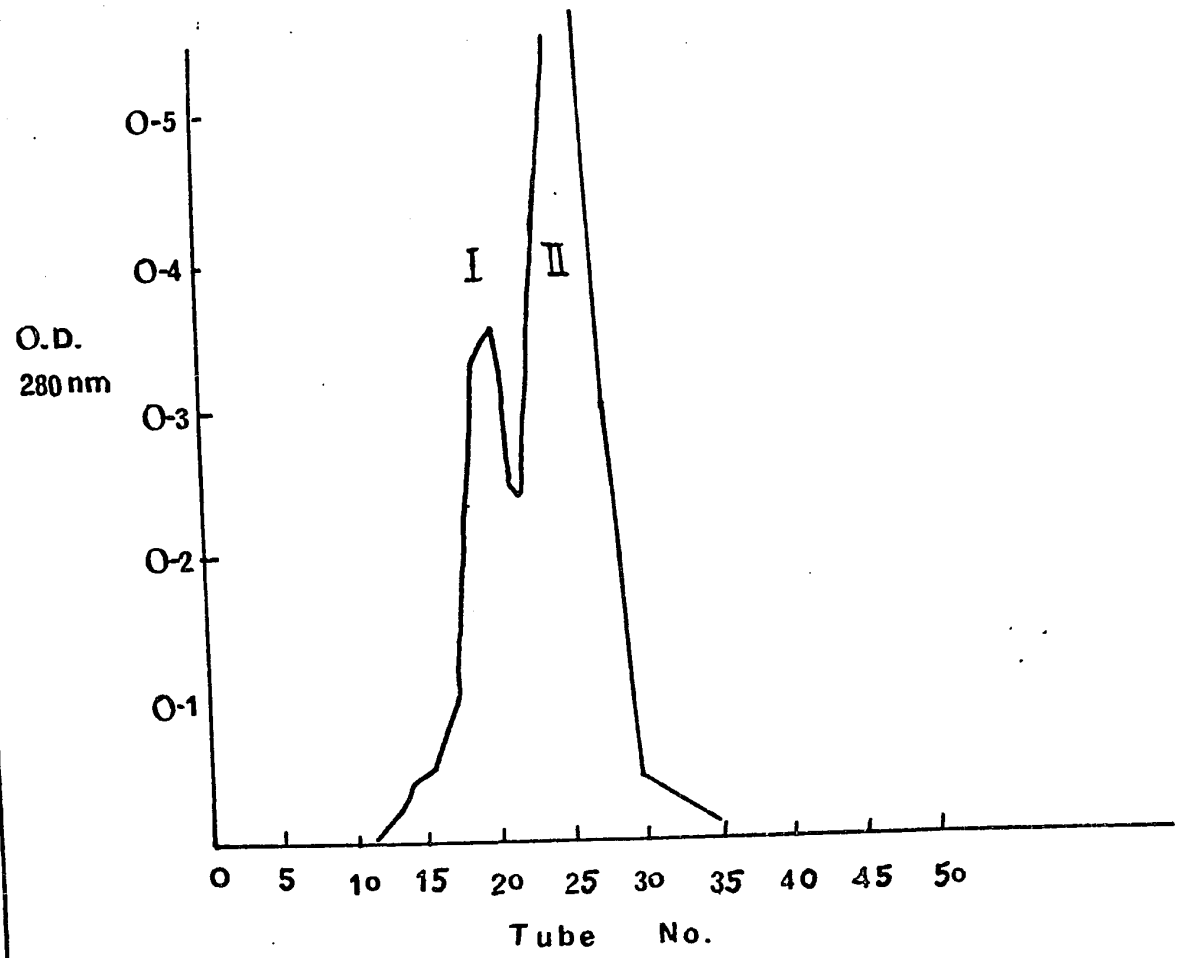
TABLE 14. Growth of S. aureus strains in fractions of CCM and of fresh medium

G 50 Peak	Fractionated Conditioned Medium		Fractionated Fresh Medium	
	S. aureus		S. aureus	
	I	II	I	II
I (1 ml + 1 ml 199)	4+	4+	4+	4+
II "	4+	4+	4+	4+
III "	+	-	4+	4+

TABLE 15. Growth of S. aureus strains in fractions from G 25 column

G 25 Peak	S. aureus	
	I	II
I (1 ml + 1 ml 199)	+	-
II "	4+	4+

Fig.4 Absorbance of fractions from Sephadex G25



(16) Sephadex G 10

Purpose:

To isolate the factor using Sephadex G10 chromatography.

Experimental:

The third peak of the G 50 column was collected and frozen-dried, reconstituted to 2 ml, and put over a column of G 10. After fractionating into four ml volumes the materials in the peaks were pooled and assayed. Three peaks were chosen (Fig. 5), peak I: tubes 4 to 8; peak II: tubes 9 to 15; and peak III: 16 to 30. 10^4 organisms were inoculated into tubes containing 1 ml of 199 and one ml of the fraction to be tested.

Results: TABLE 16.

Growth of test organisms in the presence of G 10 column fractions added to fresh medium, was seen in peak II and III but not in peak I. (Fig. 5).

Conclusion:

The void volume peak displayed the activity, indicating that the inhibitor has a molecular weight in excess of about 700.

Fig.5 Absorbance of fractions from Sephadex G 10

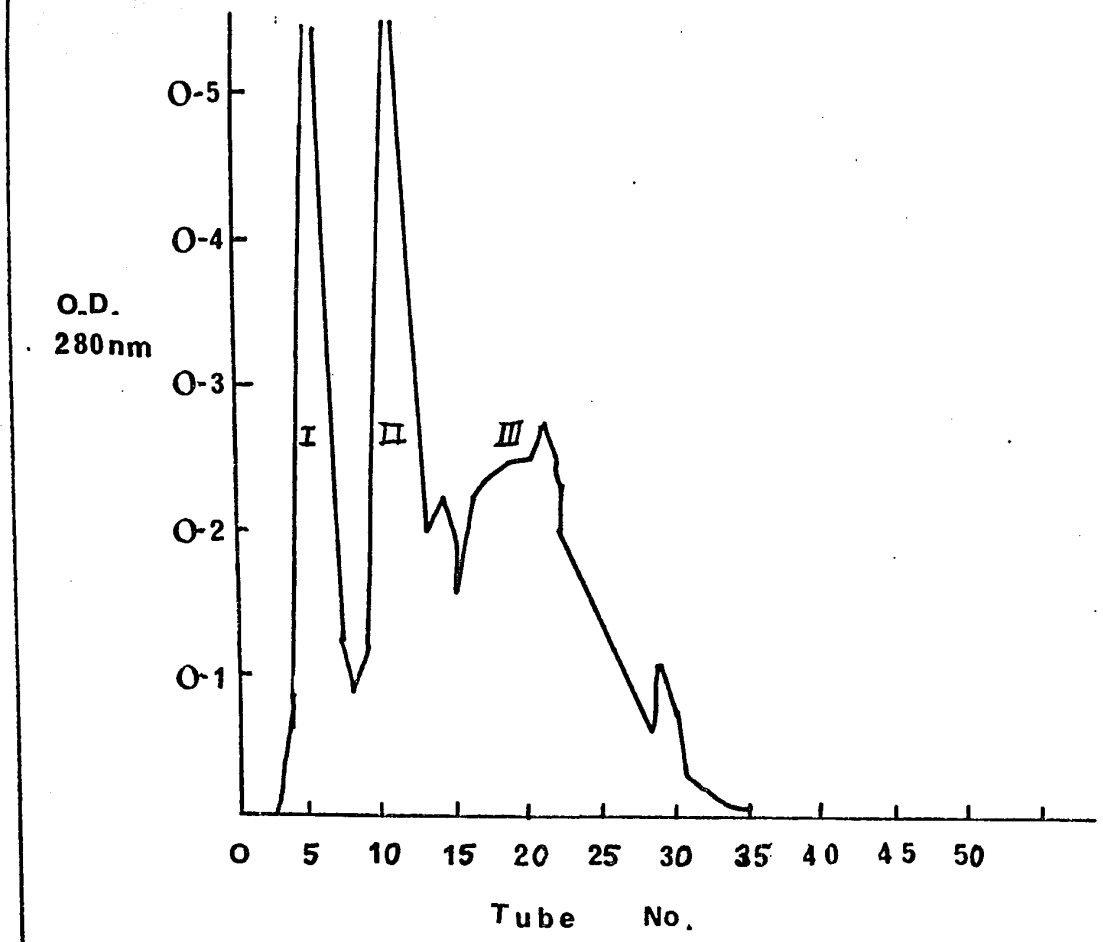


TABLE 16. Experiment 16.

Growth of S. aureus strains 2695 and 2919 in the presence of column G 10 fractions. Ten thousand organisms inoculated into each tube containing 1 ml 199 and 1 ml of the fraction to be tested.

	S. aureus	
G 10 Peak	I	II
I	2+	-
II	4+	4+
III	4+	4+

TABLE 17. Experiment 17.

Growth of 10^4 bacteria in the presence of 199 and fractions from Sephadex G 15.

	S. aureus	
G 15 Peak	I	II
I	4+	4+
II	2+	-
III	4+	4+

(17) Sephadex G 15

Purpose:

To isolate the factor using Sephadex G 15 chromatography.

Experimental:

When the third peak from the G 50 column was concentrated and passed over Sephadex G 15 the material after the void volume peak contained the activity. Three G 15 peaks (Fig. 6) were collected, peak one: tubes 5 to 9, peak two: tubes 10 to 17, peak three: tubes 18 to 30. Peaks were concentrated then mixed with fresh medium 199 for assay.

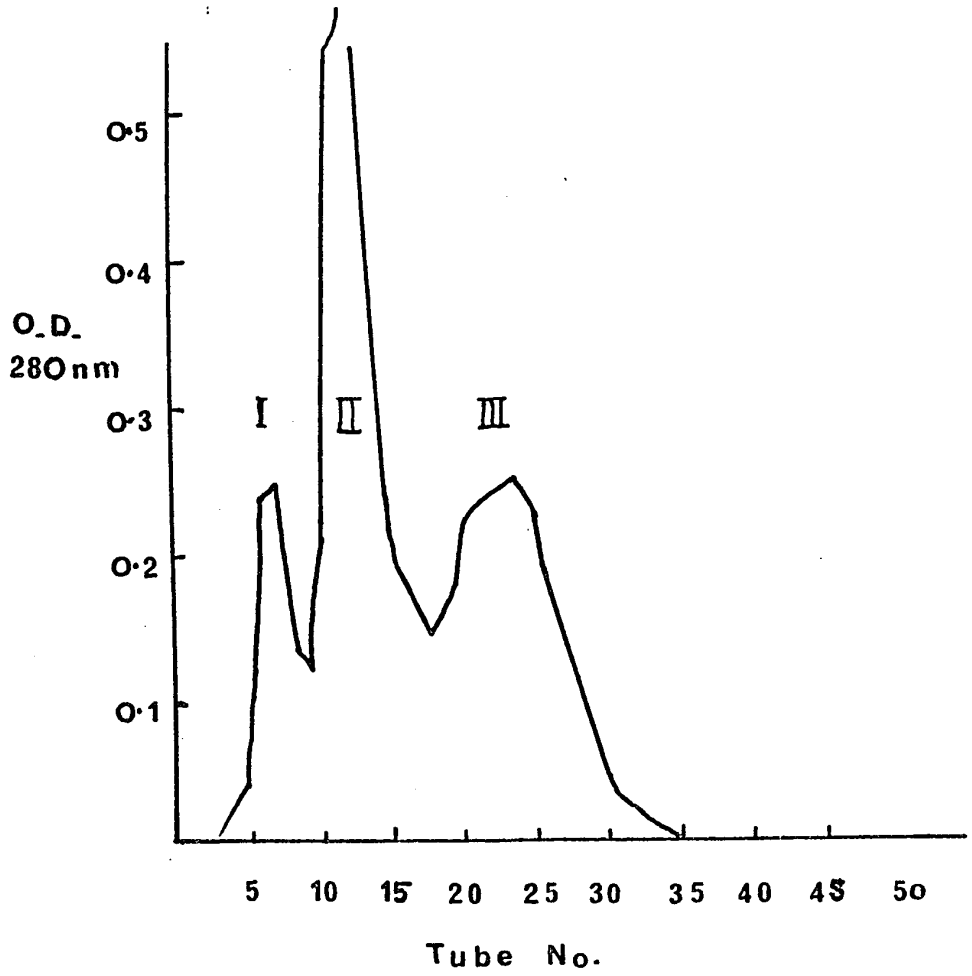
Results: TABLE 17 and Fig. 6.

Growth of the test organisms appeared in two peaks, I and III, but not peak II.

Conclusion:

The inhibitor has a molecular weight less than 1500. It must therefore have a weight between 700 and 1500.

Fig. 6 Absorbance of fractions from Sephadex G 15



D. INHIBITORY ACTIVITY AS A KETOALDEHYDE-EXPERIMENTS 18 and 19.

RATIONALE.

To identify the bacterial growth inhibitor from used HeLa cell medium it was necessary to consider its known properties for a clue to its unknown chemical nature. That it is secreted into the medium and not retained by the cell indicates that it possibly has an action against the growth of neighbours or other organisms in the environment. Thus it could control growth in a wide area around the cell that produces it. It is found in all the human cell cultures especially primary human amnion cell cultures, which suggests it is an important normal physiological substance. It is, in retrospect, not surprising that human cells should have some protection of their own against growth of invading bacteria. It is apparent that those bacteria which are inhibited in certain numbers are of lesser virulence. Others are more virulent, but even they can be inhibited if their numbers are small relative to the concentration of the factor.

On Gel Filtration it chromatographed with material of molecular weight between 700 and 1500. Its biological activity was not labile at 121°C in the autoclave.

There had been reported a substance, one of whose properties, it was argued, was to keep parasites off clams and mushrooms (304). It was found in a variety of animal tissues and was even present in human urine (91). It was named 'Retine' and was identified as a ketoaldehyde. Ketoaldehydes had been shown to inhibit the growth of E. coli reversibly and to inhibit in amounts quantitatively relative to the number of E. coli (96) (97). Such activity was suspiciously similar to that of

our factor. The similarities were greater. Retine was attached to a carrier, presumably a polypeptide, which by sephadex fractionation behaved as if it had a weight of 1000 (136). A small ketoaldehyde like methyl glyoxal for instance should be stable in heat especially if attached to a carrier by the carbonyls. The likeness of Retine to the inhibitor from HeLa cells was sufficient to warrant testing the factor for ketoaldehyde-like characteristics. The simplest indication was to study whether cysteine would reverse the inhibitory activity of the factor as it can reverse the inhibition by methyl glyoxal (96).

TABLE 18. Growth of test organism at 40 hrs, in the indicated Medium (S.aureus 2695)

MEDIUM:	i Medium 199	ii Cysteine in 199	iii ½ CCM ½ 199	iv Cysteine in ½ CCM and ½ 199
Cysteine Concentration where used				
10^{-5} M	4+	4+	-	±
10^{-4} M	4+	4+	-	+
10^{-3} M	4+	4+	-	3+

(18) The Effect of Cysteine on Growth Inhibition

Purpose:

To investigate whether the inhibitory factor has ketoaldehyde properties by attempting to reverse the activity with cysteine.

Experimental:

Contained in a volume of 0.1 ml 10^4 organisms (S. aureus of phage group II strain 2695) were inoculated into tubes containing 2 ml 199 (i), 2 ml 199 and added cysteine to 10^{-3}M (ii), 1 ml 199 and 1 ml of conditioned medium (iii), and 1 ml 199 with 1 ml conditioned medium and added cysteine to 10^{-3}M (iv). Two other series with cysteine added to 10^{-4}M and 10^{-5}M were also set up.

Results: TABLE 18.

Growth occurred in Medium 199 alone, and in Cysteine and 199. Medium containing CCM inhibited the growth of these organisms but where Cysteine was added to CCM growth appeared relative to the amount of Cysteine added.

Conclusion:

The addition of cysteine to the growth medium appeared to block the inhibition. At these concentrations this phenomenon is not immediate but is seen after forty hours. The possibility that the inhibitor could be a ketoaldehyde, is not ruled out.

TABLE 19. The effect of methyl glyoxal on growth of S. aureus strains from phage groups I to IV (10^5 organisms).

methyl glyoxal concentration	time of observation	Phage Group I	II	III	IV
0.35×10^{-2} M	5 hrs	-	-	-	-
10^{-3} M	"	+	-	+	-
10^{-4} M	"	+	-	+	-
10^{-5} M	"	+	-	+	-
10^{-6} M	"	+	-	+	-
0.35×10^{-2} M	15	-	-	-	-
10^{-3} M	"	2+	±	2+	+
10^{-4} M	"	2+	2+	2+	2+
10^{-5} M	"	2+	2+	2+	2+
10^{-6} M	"	2+	2+	2+	2+
Control	15 hrs	4+	4+	4+	4+

(19) Action of Methyl Glyoxal On Growth Of Organisms

Purpose:

To evaluate whether the type of growth inhibition by methyl glyoxal mimics the action of the bacterial inhibitor.

Experimental:

S. aureus strains from phage groups I, II, III and IV were inoculated into medium 199 containing methyl glyoxal in concentrations of 0.35×10^{-2} to 0.35×10^{-6}. Growth was observed at 5 hrs and 15 hrs. Phage group III is virulent and IV is less virulent as noted in Table 3 and as explained in the Discussion.

Results: TABLE 19.

At 5 hrs a distinct difference was seen in the growth response of organisms from Phage Groups I and III and Groups II and IV. II and IV were more inhibited. At 15 hrs this distinction is not discernible except at a concentration of 0.35×10^{-3} M Methyl Glyoxal.

Conclusion:

Methyl glyoxal inhibits, in five hours, all strains at 0.35×10^{-2} M but at 10^{-3} M strains from group II and IV are more inhibited relative to group I and III. At 0.35×10^{-4} or less the latter is true only for the first 5 hours after which the organisms can overcome the effect of methyl glyoxal. The specificity of the action of methyl glyoxal resembles that of the inhibitor but the short period of inhibition suggests that the active portion of the latter is not itself methyl glyoxal but could be a more active ketoaldehyde.

E. ANALYSIS OF STRUCTURE - EXPERIMENTS 20 to 35

(20) Isolating The Factor

Purpose:

To attempt to remove a ketoaldehyde from a carrier by arsenolysis.

Experimental:

As a ketoaldehyde is highly reactive for -SH or $-NH_2$ groups As_2O_3 should compete for them and tend to liberate free ketoaldehyde (95). The active fraction following Sephadex G 25 was collected, filtered sterile and lyophylized. Its dry residue was dissolved in 1.5 ml sterile H_2O and split into 3 portions of 0.5 ml each. The first was added to 4 ml fresh medium 199 to test for inhibitory activity. The second and third were added to 4.5 ml methanol each and to the third was also added 0.01 gm As_2O_3 which was insoluble. Both methanol mixtures were agitated at 37°C for 24 hrs and then were centrifuged. A precipitate formed in each tube. The clear methanol supernatants were removed. The one from the tube with As_2O_3 was yellowish brown, and in a sample of it no arsenate or arsenic was detected by lead acetate or by hydrogen sulfide respectively. Both supernatants were heated to 45°C in open tubes to drive off the methanol. A remarkable pleasant odour was noted in the yellow residue. The odour had a sweet yet pungent nature reminiscent of the smell of an orchard in autumn where apples lie rotting on the ground. The two residual volumes were each added separately to 4 ml medium and all were filtered sterile. Two aliquots of 2 ml each were taken from each of these three samples,

and were inoculated with 10^5 organisms of S. aureus strains 2919 and 2695 in the two aliquots. On the occasions when this experiment was repeated further samples were used to test the growth of N. meningitidis and E. coli strains.

Results: See TABLE 20.

Conclusion:

The inhibitory factor in the G 25 fraction is in a form insoluble in methanol but As_2O_3 can free the active agent which becomes soluble in methanol. Colour is similar to ketoaldehyde solutions.

TABLE 20. Experiment 20.

Inhibitory activity of extracts from partially purified active material, assayed in medium 199 against 10^5 bacteria.

STRAIN	GROWTH IN MEDIUM WITH			
	199 only	Water soluble material	CH_3OH soluble material	CH_3OH soluble material after As_2O_3
S. aureus				
2695 group II	4+	-	3+	-
2919 group I	4+	3+	4+	3+
N. meningitidis				
1027 type A	4+	-	4+	-
Slat type X	4+	-	4+	-
604 type A	4+	2+	4+	3+
247 type X	4+	4+	4+	3+
E. coli				
014	4+	-	4+	-
026	4+	3+	4+	3+

TABLE 21. Effect of extracts from non inhibitory used medium on growth of 10^5 S. aureus test organisms in Med. 199.

S. aureus Strain	Fresh medium 199 only	Water soluble material	CH_3OH soluble material	CH_3OH soluble after As_2O_3
less V	4+	4+	4+	4+
V	4+	4+	4+	4+

(21) A Control For As_2O_3 Treatment

Purpose:

To test the effect of As_2O_3 on a fraction of conditioned medium not containing the inhibitor, in order to exclude the possibility that As_2O_3 itself is causing an artifact.

Experimental:

Separate cultures of HeLa cells in 199 had occasionally been found contaminated with PPLO and the conditioned medium had shown no antibacterial activity (Table 10). This medium was lyophilized and fractionated over G 50 and G 25 columns and the fraction corresponding to the actively inhibitory fraction from normal conditioned medium was collected. It was treated as was the active material in experiment 20. Three parts were separated, two of which were mixed with methanol. As_2O_3 was added to one of these latter and they were both agitated for 24 hrs at 37°C. Following separation of the clear methanol extracts and their evaporation to a residual volume, growth of bacteria was tested in medium mixed with these residues, and rendered sterile by filtration.

Results: TABLE 21.

Under no conditions was inhibitory activity found in material from HeLa cells with PPLO. The test organism was capable of growing equally well in fresh medium 199, 199 with water soluble column fraction, 199 with methanol soluble material and 199 with methanol soluble material after As_2O_3 treatment.

Observation:

The CH_3OH - As_2O_3 extract is not yellow but clear, the UV spectrum is in Fig. 8.

Conclusion:

Where inhibitory activity is lacking in used medium there is no activity in the CH_3OH - As_2O_3 extract. Therefore As_2O_3 does not create any artifact from used medium responsible for the growth inhibition in experiment 20. The yellow colour of the extract in experiment 20 seems to correlate with antibacterial activity as its absence correlates with no activity.

(22) Electronic Spectra of Methanol Extracts

Purpose:

To observe the electronic spectra of the actively inhibitory methanol-As_2O_3 extract for evidence of the presence of a ketoaldehyde.

Experimental:

The absorption spectra of the methanol-As_2O_3 extracts of experiments 20 and 21 were recorded. Samples of the solutions were added to methanol and read against a methanol blank. For comparison solutions of the mono-carbonyl compound methyl ethyl ketone and the α-dicarbonyl compound methyl glyoxal (pyruvaldehyde) were put in methanol. A sample of each of these solutions was made alkaline by the addition of methanol-NaOH solution. Dicarbonyls in alkali are known to form the conjugated enolate ion and show a characteristic absorption shift from around 270 nm to 290 nm, and an increase in the absorbance. Preparations of retine (91) (92) displayed this effect. (See Discussion for a fairly complete explanation of the UV spectra of ketoaldehydes).

Results:

Absorbance peaks of methanol-As_2O_3 extracts of Exp. 20 are around 310 nm and 330 nm but are not too sharp (Fig. 7). Only End Absorption is evident from the same extract taken from the inactive starting material used in Exp. 21 (Fig. 8). Alkali does not alter the absorbance of the latter while it does give rise to an increase at 295 nm for the active material. While methanol solutions of methyl glyoxal display a peak at 280 nm, alkali shifts the peak to 295 nm and raises it (Fig. 9).

Methyl ethyl ketone remains at a peak near 270 nm neutral or alkaline. (Fig. 10).

Conclusion:

Methyl glyoxal and the methanol-arsenate extract both show a similar spectral shift with alkali, indicating that the latter probably contains a dicarbonyl compound.

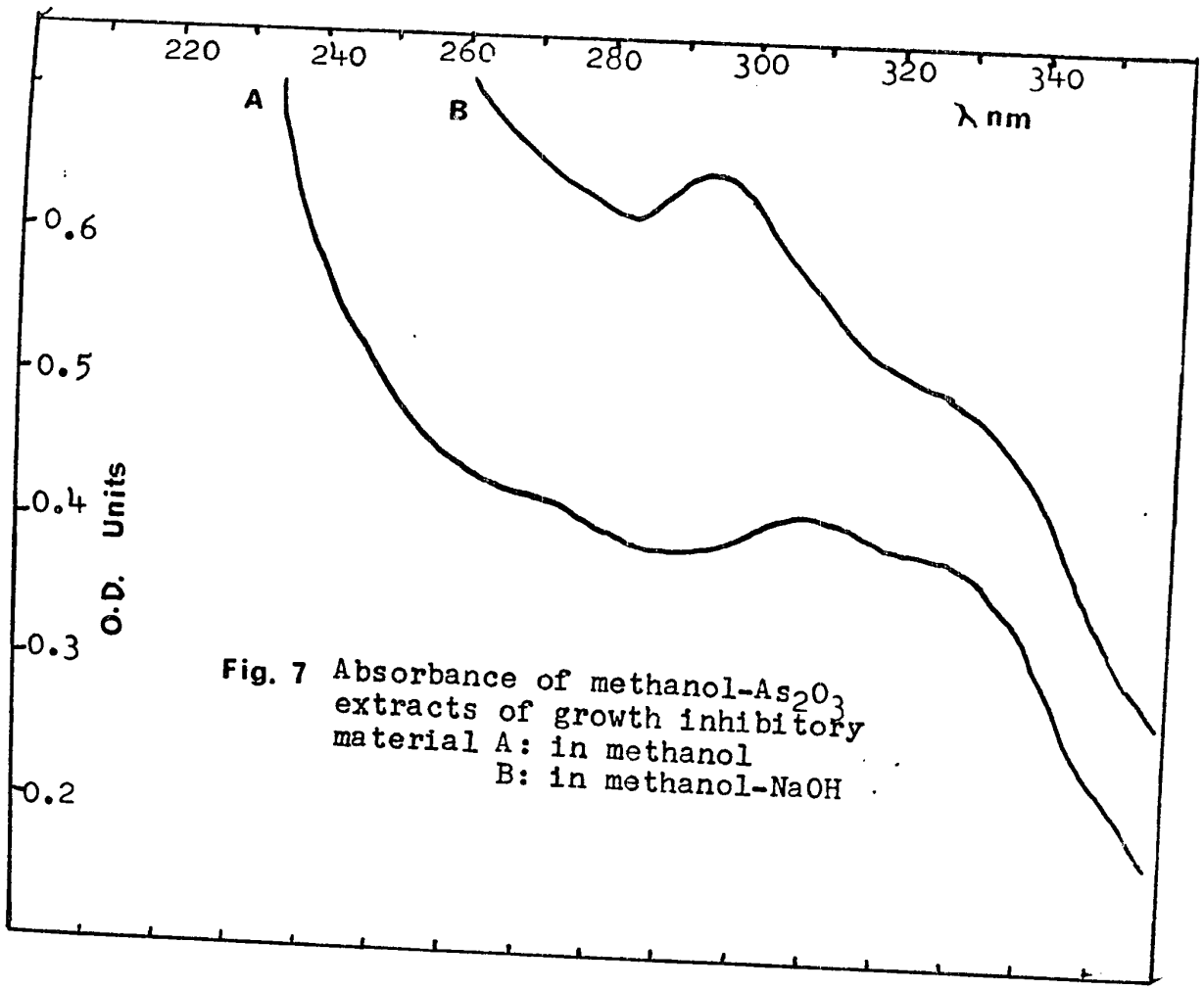


Fig. 7 Absorbance of methanol-As_2O_3 extracts of growth inhibitory material A: in methanol
B: in methanol-NaOH

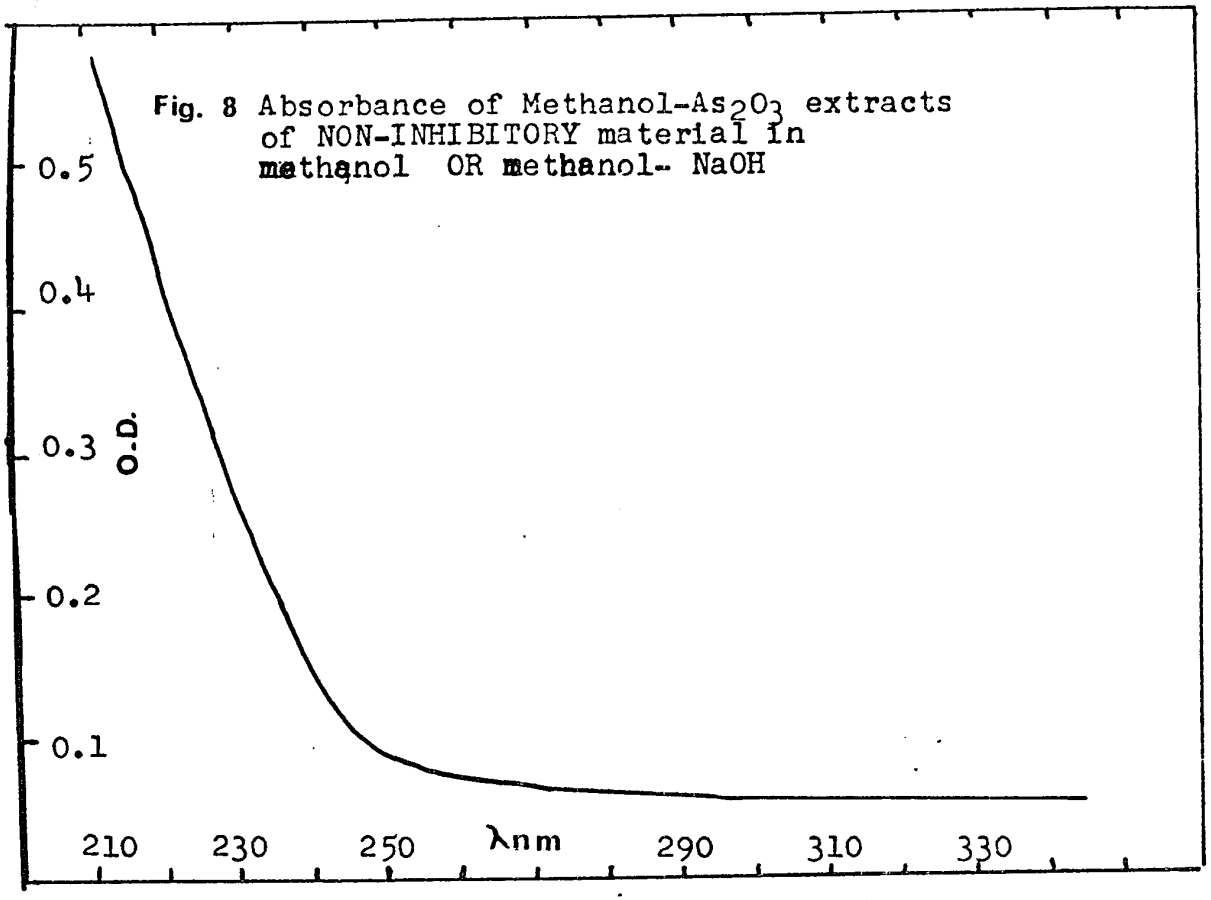


Fig. 8 Absorbance of Methanol-As_2O_3 extracts of NON-INHIBITORY material in methanol OR methanol- NaOH

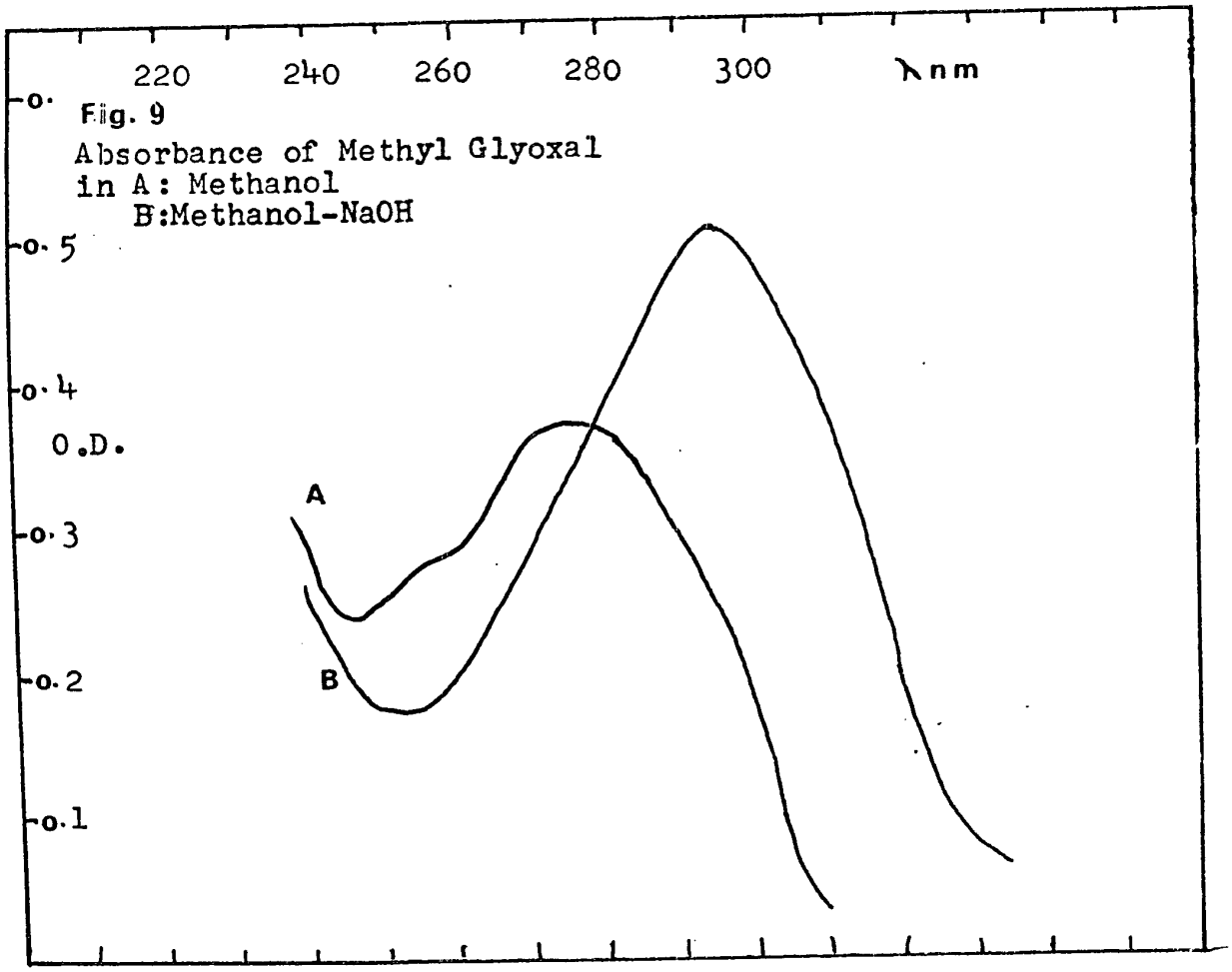


Fig. 9
Absorbance of Methyl Glyoxal
in A: Methanol
B:Methanol-NaOH

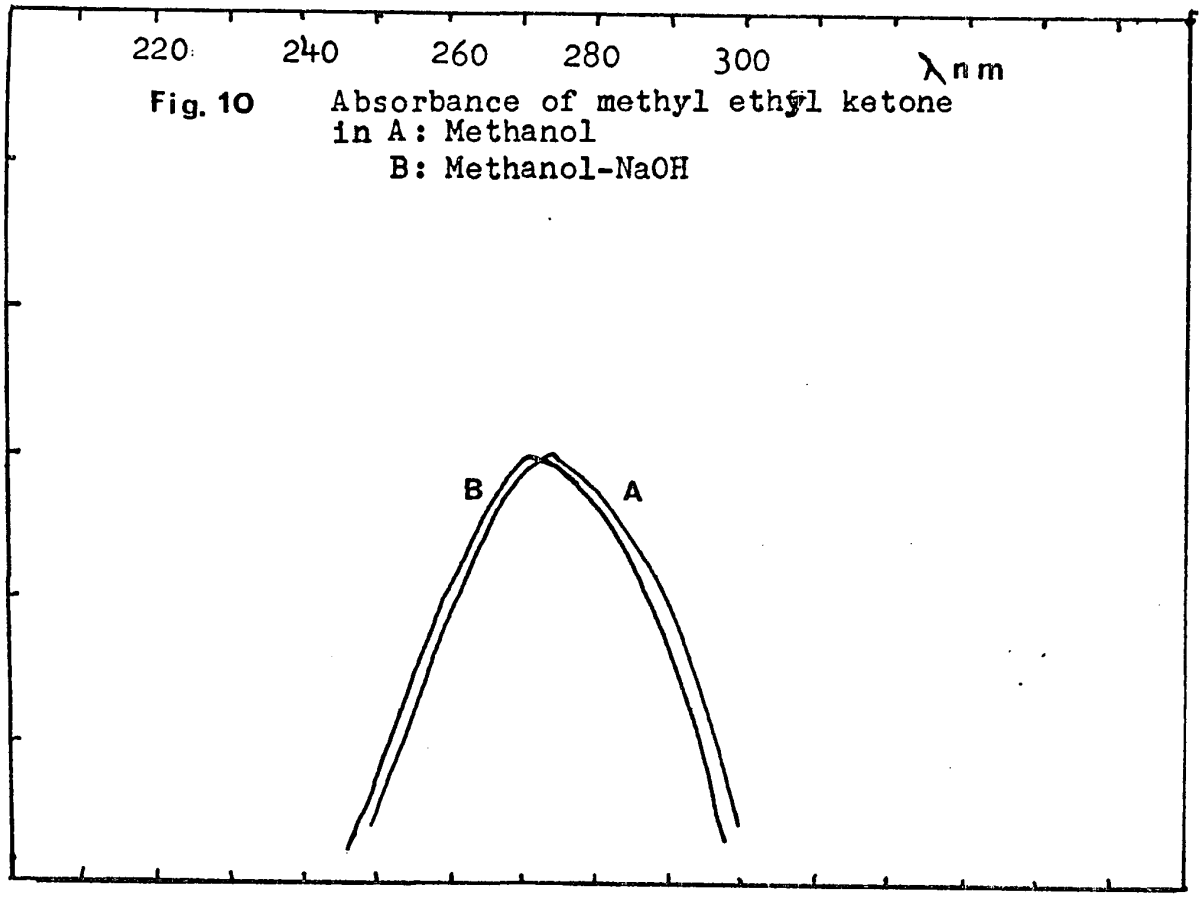


Fig. 10 Absorbance of methyl ethyl ketone in A: Methanol
B: Methanol-NaOH

(23) Further Separation

Purpose:

To remove impurities from the supposed ketoaldehyde in the methanol-arsenate extract.

Experimental:

Ethanol is slightly less polar than methanol and may not solublize substances of a polar nature soluble in methanol. Therefore a change of solvents was made to separate some solutes from others. The methanol was driven off the extract by heating to about 80°C. Since ketoaldehydes can be oxidized to hydroxy acids, especially under alkaline conditions, some acid was added to the methanol solution to bring it to 0.01 N HCl before the evaporation, in the hope of minimizing any oxidation. To the residual volume 2 ml of ethanol was added and the mixture was held at 4°C for 24 hrs. A white precipitate had come out but the ethanol retained the yellow colour (Fig. 13). The clear ethanol was removed, dried, and its residue was redissolved in 0.001 N HCl, poured through a column of DEAE cellulose (5" x ½") in 0.001 N HCl to remove any acids formed from oxidation. The eluate was collected and the absorption spectrum of all the tubes was recorded.

Results: Fig. 11, Fig. 12, and Fig. 13.

Conclusion:

The absorption is more sharp at 285 nm than the crude extract absorption, indicating that the compound could have been separated from other UV absorbing materials and still retained the dicarbonyl intact. The spectral shift in alkali is not as extensive as in Exp. 22 suggesting that less enolate anion can be formed after the treatment given here.

Fig. 11 Absorbance @ 285 nm of Eluate

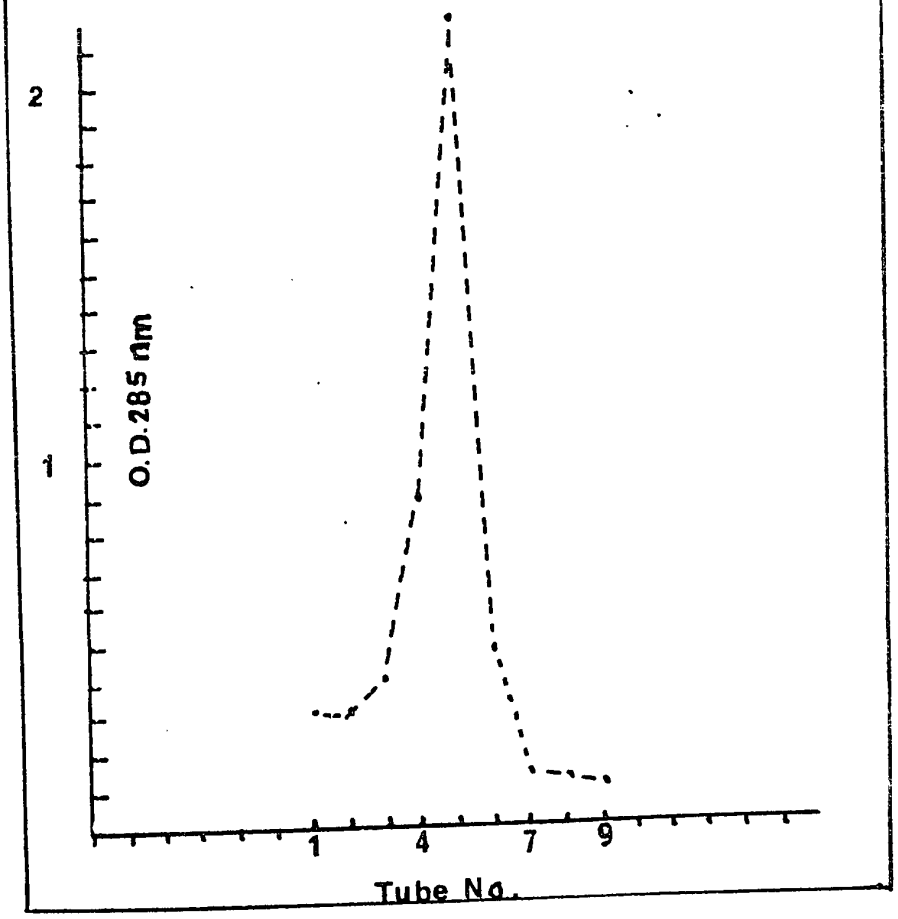
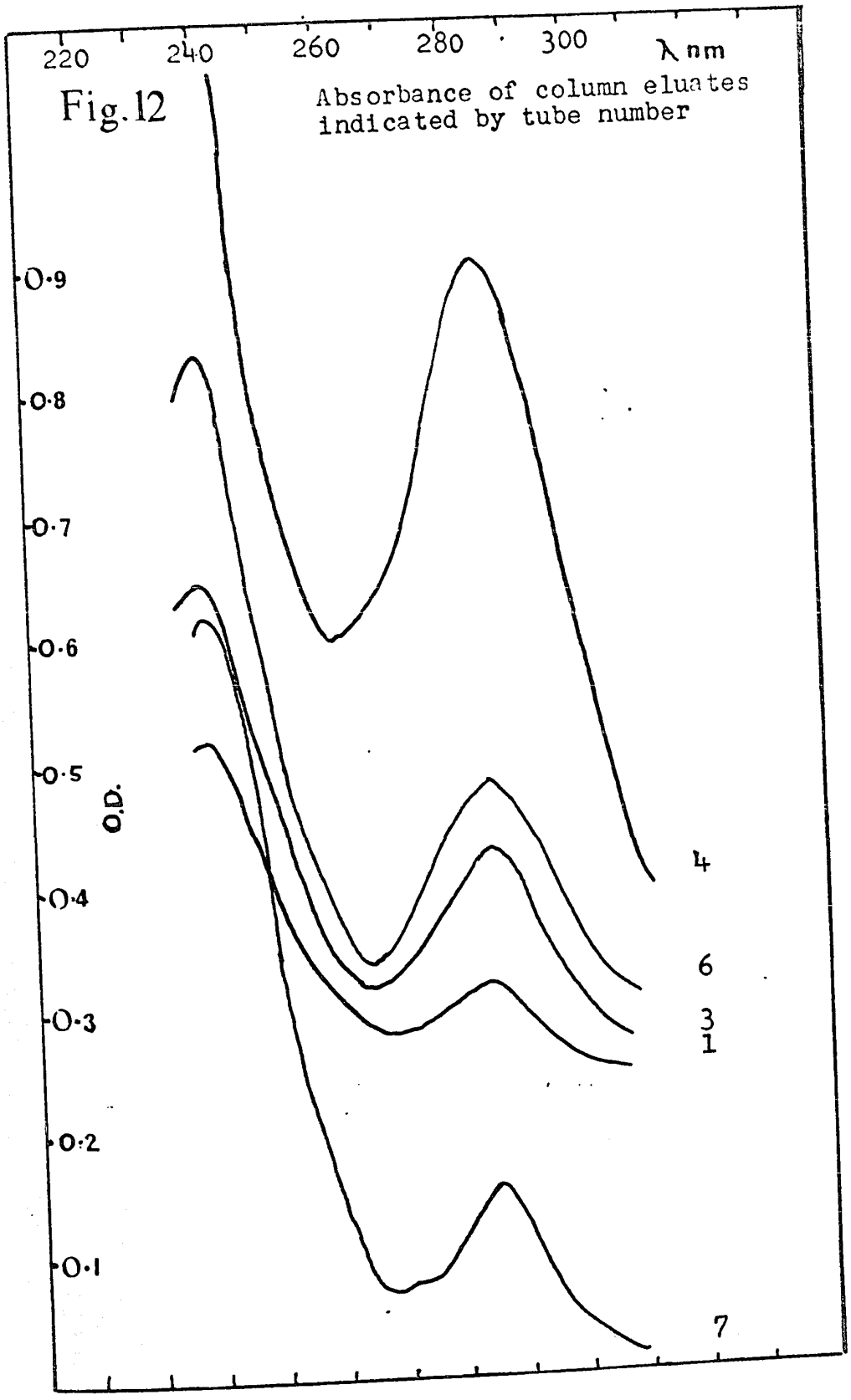


Fig. 12


220
240
260
280
300
λ nm
Absorbance of column eluates indicated by tube number
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
O.D.
4
6
3
1
7

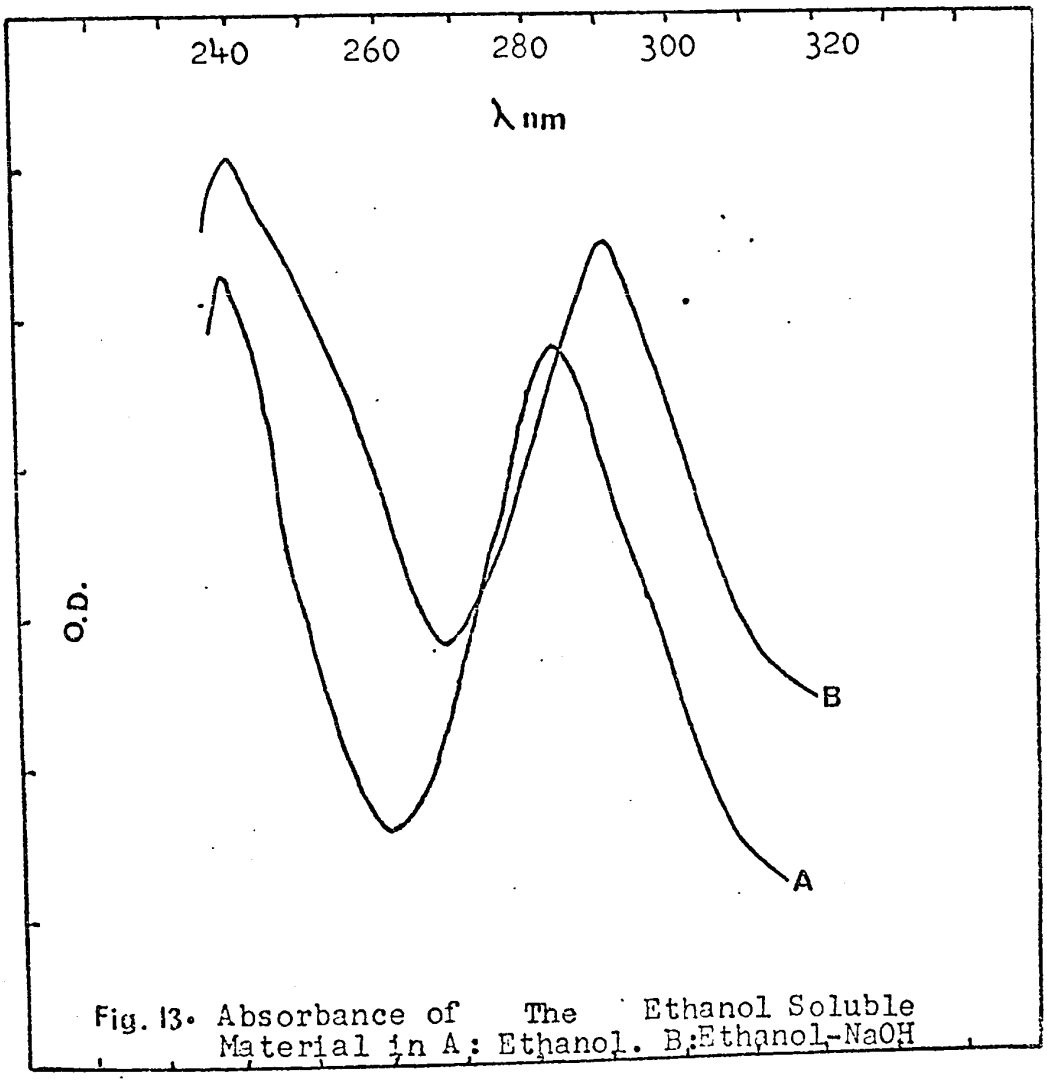


Fig. 13. Absorbance of The Ethanol Soluble Material in A: Ethanol. B:Ethanol-NaOH

TABLE 22. Growth of S. aureus strains from phage groups I and II in test medium.

Tube Assayed	Group I		Group II	
1	4+	4+	4+	4+
2	4+	4+	4+	4+
3	4+	2+	2+	2+
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	4+	2+	-	-
8	4+	4+	-	-
9	2+	4+	-	-

(24) Activity of Cleaned Fractions

Purpose:

To assay the fractions from the DEAE cellulose column of Exp.23 for antibacterial activity.

Experimental:

The light absorbing material was collected in 9 tubes. Each sample was filtered sterile and 0.2 ml of each was added to 1 ml aliquots of med. 199. Into each tube were inoculated 1000 organisms. Virulent and less virulent strains were tested and their growth was observed after 18 hrs. Tubes were assayed in duplicate.

Results: TABLE 22

The material from tube fractions 4, 5, and 6 seemed to be in such a concentration as to inhibit the growth of even the virulent strain. Activity was also present in tube fractions 7, 8, and 9, but not enough to inhibit the virulent strain.

Conclusion:

The cleaned compound, after alcohol extraction and column passage demonstrates the inhibitory activity noted previously in conditioned medium.

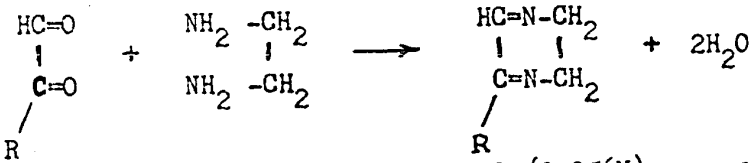
(25) Reaction with Ethylene Diamine

Purpose:

To identify the dicarbonyl compound as an α-dicarbonyl compound by reaction with ethylene diamine (EDAM).

Experimental:

Only vicinal carbonyl compounds react with EDAM to form yellow coloured dihydropyrazine compounds. Therefore EDAM in methanol (0.05M) was



mixed in equal volume with methyl glyoxal (0.056M) as a control. Methyl glyoxal, 0.056M, has an optical density of 0.320 at 276 nm. Therefore the methanol-As_2O_3 extract was diluted with methanol to this O.D. 276 nm and mixed in equal volumes with 0.05 M EDAM. In addition the cleaned ethanol extract after DEAE cellulose chromatography (Exp. 23) was tested. Spectra were recorded in water solutions at various H^+ ion concentrations after evaporating the methanol off.

Results: Fig. 14.

Curve A shows that the reaction product of methyl glyoxal and EDAM has 5 peaks; at 344 nm, 376 nm, 405 nm, 288 nm, and 228 nm. Water at pH 4 (Curve B) blurred the bands above 300 nm to absorb at the position of the maximum band 376 nm. The 288 nm band remained the same while the 228 nm absorption increased in intensity. More ionized solvent (NaOH added) lowered the intensity and wavelength of the 378 nm band to 340 nm. The 288 nm absorption remained unchanged while the intensity of the 228 nm absorption became very high (Curve C).

The methanol-As_2O_3 extract and EDAM gave a product displaying a band at 222 nm, a band at 288 nm, and two peaks at 328 nm and 383 nm which shift to 297 nm and 332 nm on slight acidification. Further acidification eliminated the band at 332 nm and depressed the intensity of the 297 nm peak: (Fig. 15).

The purified extract after DEAE column treatment gave a reaction product with EDAM showing absorption at 228 nm, 280 nm and weak peaks at 322 nm and 370 nm with 1 N HCl. In alkali the 228 nm absorption rose very high in intensity, the peak at 280 nm disappeared leaving visible a slight peak at 275 nm, and a large absorption took place at 322 nm: (Fig. 16).

Conclusion:

Methyl glyoxal and the unknown ketoaldehyde are not the same compound yet both yield similar types of derivatives on reaction with EDAM thus demonstrating the vicinal carbonyl structure. Dihydropyrazine compounds display marked shifts in their absorption spectra due to solvent changes in polarity and pH.

(See Discussion for an analysis of dihydropyrazine spectra and an explanation of Figs. 14, 15, and 16).

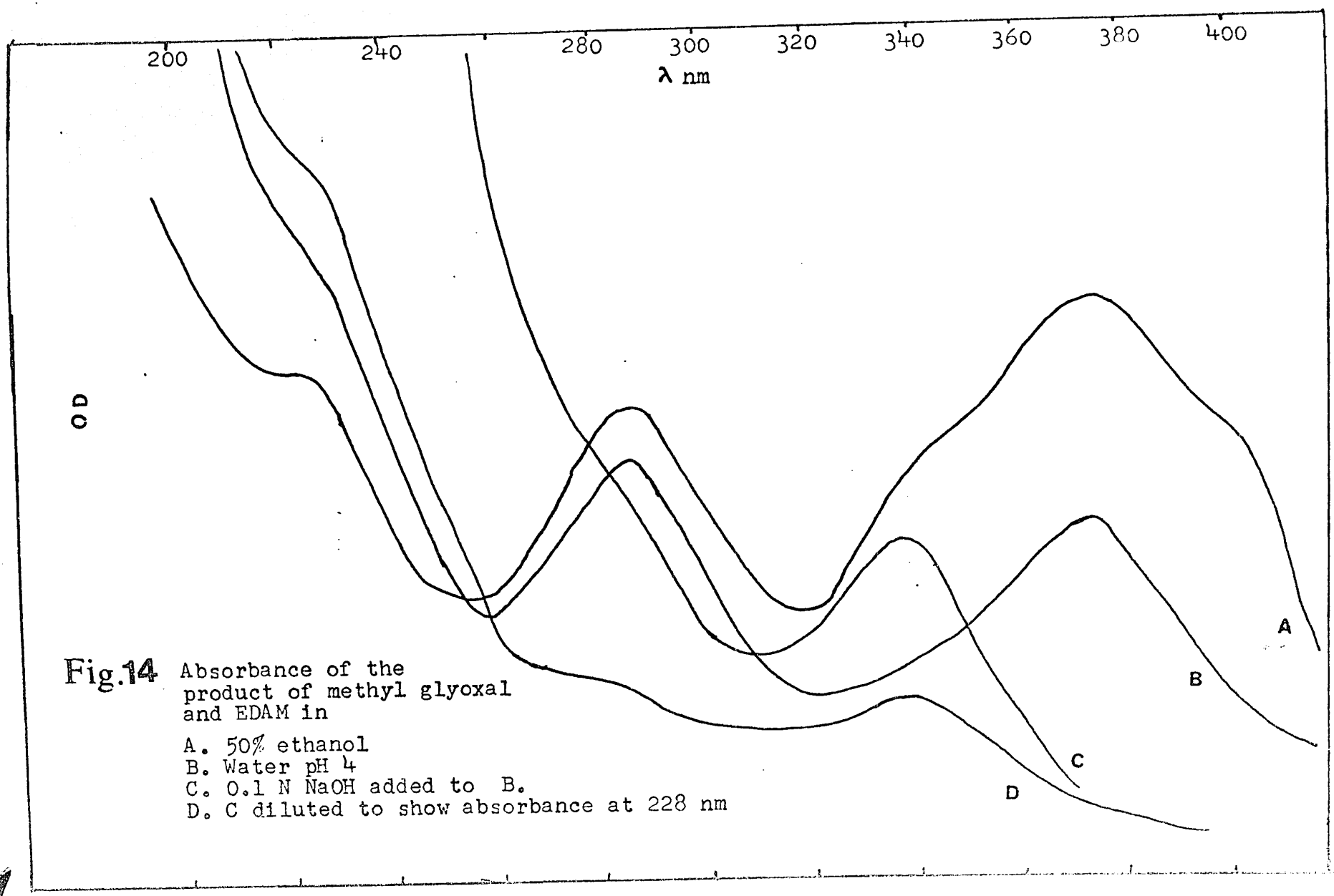


Fig.14 Absorbance of the product of methyl glyoxal and EDAM in

A. 50% ethanol
B. Water pH 4
C. 0.1 N NaOH added to B.
D. C diluted to show absorbance at 228 nm

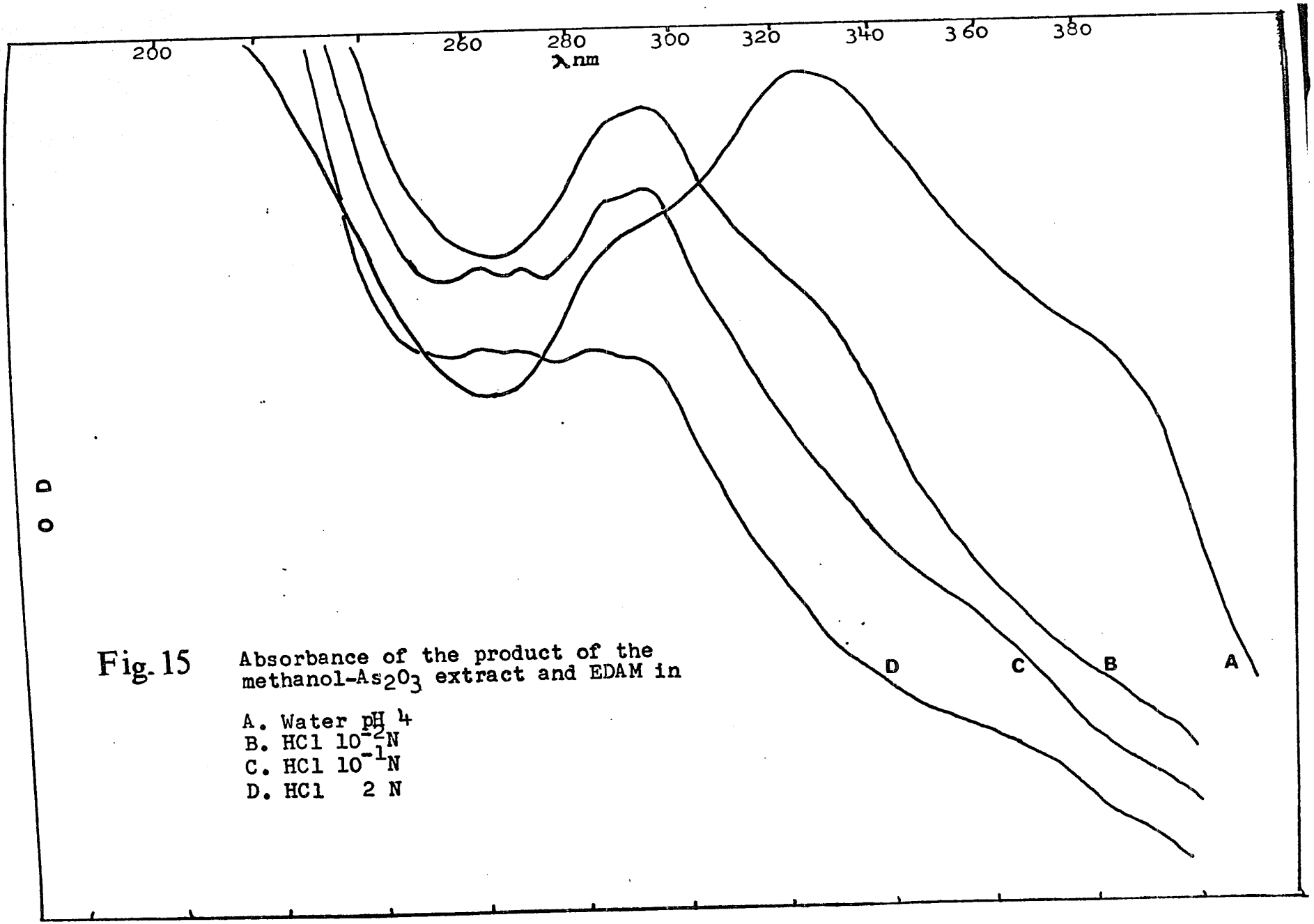


Fig. 15 Absorbance of the product of the methanol-As_2O_3 extract and EDAM in

A. Water pH 4
B. HCl 10^{-2}N
C. HCl 10^{-1}N
D. HCl 2 N

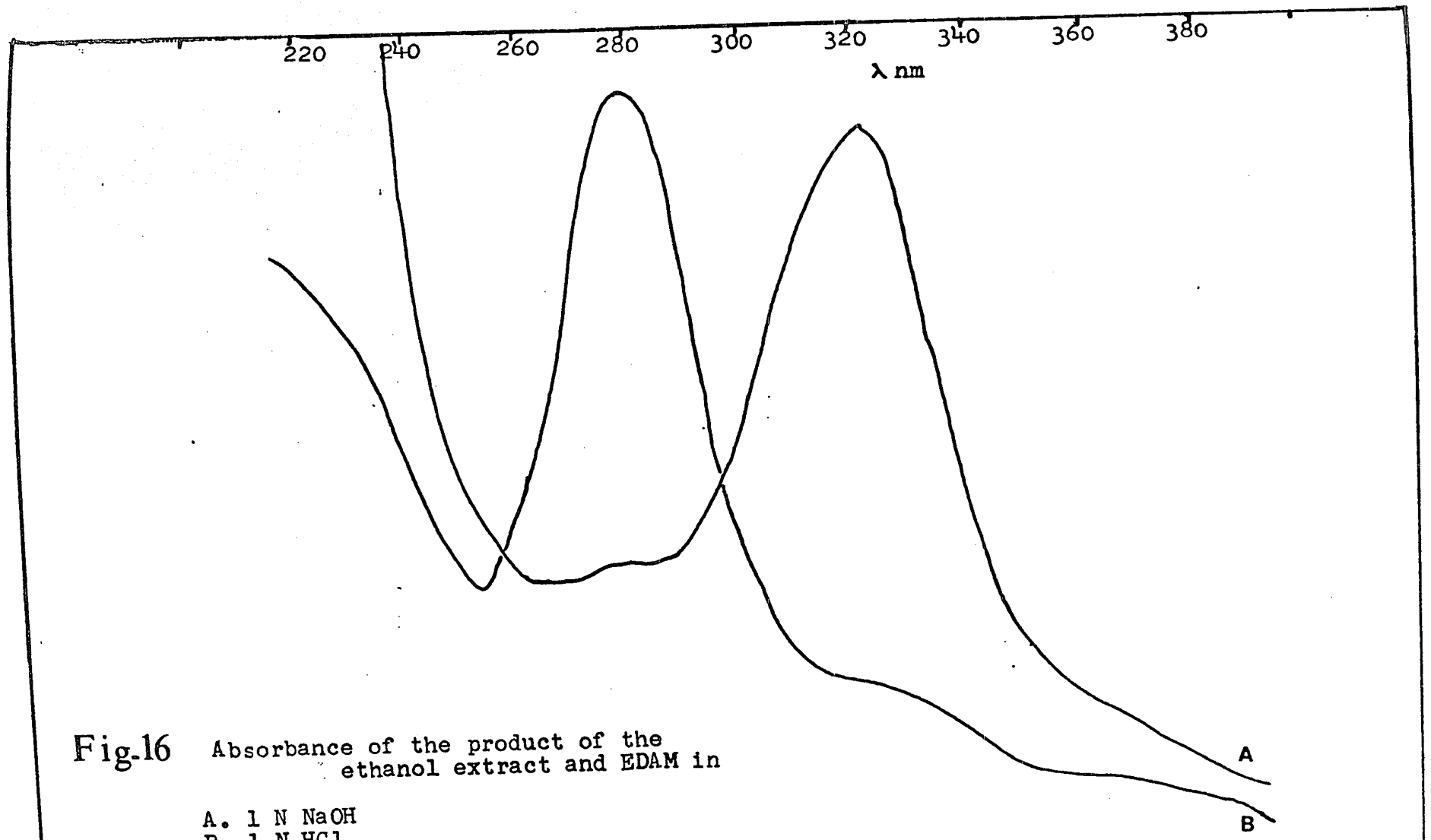


Fig.16 Absorbance of the product of the ethanol extract and EDAM in

A. 1 N NaOH
B. 1 N HCl

(26) Formation of a Dinitrophenylhydrazine Derivative.

Purpose:

To isolate the ketoaldehyde as the dinitrophenylhydrazine derivative.

Experimental:

The cleaned ketoaldehyde from the column fractionation (exp. 23) had been eluted in 0.001 N HCl in water. One ml of this (from Tube 5) was shaken with 2 ml of chloroform. The yellow colour stayed in the water phase. $CHCl_3$ was removed and to the separate water phase was added 0.1 ml glacial acetic acid. The tube was kept in an ice bath and 0.1 ml cold DNPH reagent was added; (Two gm of DNPH dissolved in 100 methanol with 4 ml conc. H_2SO_4). After a minute in the cold, dark red crystals began to form. After 15 minutes many crystals had precipitated. They were washed 8 times at the centrifuge with 0.01 N HCl to remove any unreacted DNPH, then twice with 2N Na_2CO_3 to remove any trace of a keto acid derivative; however no brown colour was observed in this wash. Washing resumed in 0.01 N HCl and H_2O. A sample was applied to a thin-layer plate which was developed in methanol-ethyl acetate 5:1. The melting point was determined and the remaining crystals were dried under vacuum.

Results:

The crystals chromatographed as only one spot visible under white or UV light. Only 0.0032 gm of crystals were retrieved. Their melting point was 220°C. (DNPH crystals are orange and melted at 200°C).

Conclusion:

The rapidity with which the derivative formed at 0°C indicates the ketoaldehyde was present and not a hydroxyaldehyde as with sugars which give the osazone only with heating. It appeared that only one compound was isolated.

(27) Confirmation that As_2O_3 frees the Ketoaldehyde from other material

Purpose:

To confirm that the antibacterial activity is identifiable as a keto aldehyde carried on larger material.

Experimental:

Exp. 20 demonstrated that the antibacterial activity is at first methanol insoluble, then soluble after As_2O_3 treatment. In this present experiment 1 ml of methanol was added to the lyophylized peak I of a G 25 fractionation. After 8 hrs at 37°C insoluble matter was separated and saved. The methanol was evaporated by heating and 1 ml absolute ethanol was added to the residual volume. At 0°C a precipitate formed in a few hours. The supernatant from this was removed, diluted in $\frac{1}{2}$ with 2N HCl, added to an equal volume of water and mixed with DNPH reagent. No precipitate was formed even after 4 days.

The methanol insoluble material formerly separated was incubated with As_2O_3 and methanol for 8 hrs at 37°C. The supernatant was removed, evaporated by heating, was resolublized in ethanol, diluted to 4 volumes of 0.5N HCl and mixed with DNPH reagent. A red crystalline precipitate was observed to form in a few minutes at 0°C.

Results:

The red crystals melted at 220°C.

Conclusion:

The antibacterial activity attached to a carrier as observed in exp.20, can be correlated with the presence of a ketoaldehyde (isolated as the osazone) attached to a carrier in a similar sample. When attached to a

carrier both the activity and the ketoaldehyde are insoluble in methanol but As_2O_3 can liberate both from the carrier to be methanol soluble. It seems at least that the activity and the ketoaldehyde have similar physical properties. However without testing the carrier residue for activity it cannot be rigorously concluded that all the activity had these properties.

(28) Electronic Spectra of the Osazone

Purpose:

To examine the osazone by its electronic absorption spectrum.

Experimental:

A sample of crystals of the DNPH derivative of the unknown ketoaldehyde was prepared as in Exp. 26. The crystals were thoroughly washed free of DNPH and were dissolved in methanol. The electronic spectrum was recorded of the resulting orange solution. The same solution was diluted and made alkaline with KOH-Methanol. The absorption of the blue colour was recorded. A DNPH derivative of methyl glyoxal was made and also studied for comparison.

Results: Fig. 17 and Fig. 18.

The neutral solution of the unknown osazone has absorption maxima at 395 nm and 423 nm while the alkaline solution displays one maximum at 566 nm. Methyl glyoxal osazone in neutral solution absorbs at 393 nm and 436 nm with a separate peak at 375 nm; in alkali the maximum is 561 nm.

Conclusion:

The blue colour in alkali, λ max. 566 nm, indicates the presence of a bis-DNPH derivative confirming the presence of an α dicarbonyl compound. The two conjugated imino linkages in bis-DNPH derivatives are responsible for the absorption at 395 and 430 nm in neutral solution. However the lack of absorption below 380 nm, that is from 350 to 380 nm, indicates further a lack of saturation and therefore the presence of double bonds conjugated with the imino linkages, (See Discussion). The methyl group of methyl glyoxal osazone shows its saturation by a peak at 375 nm.

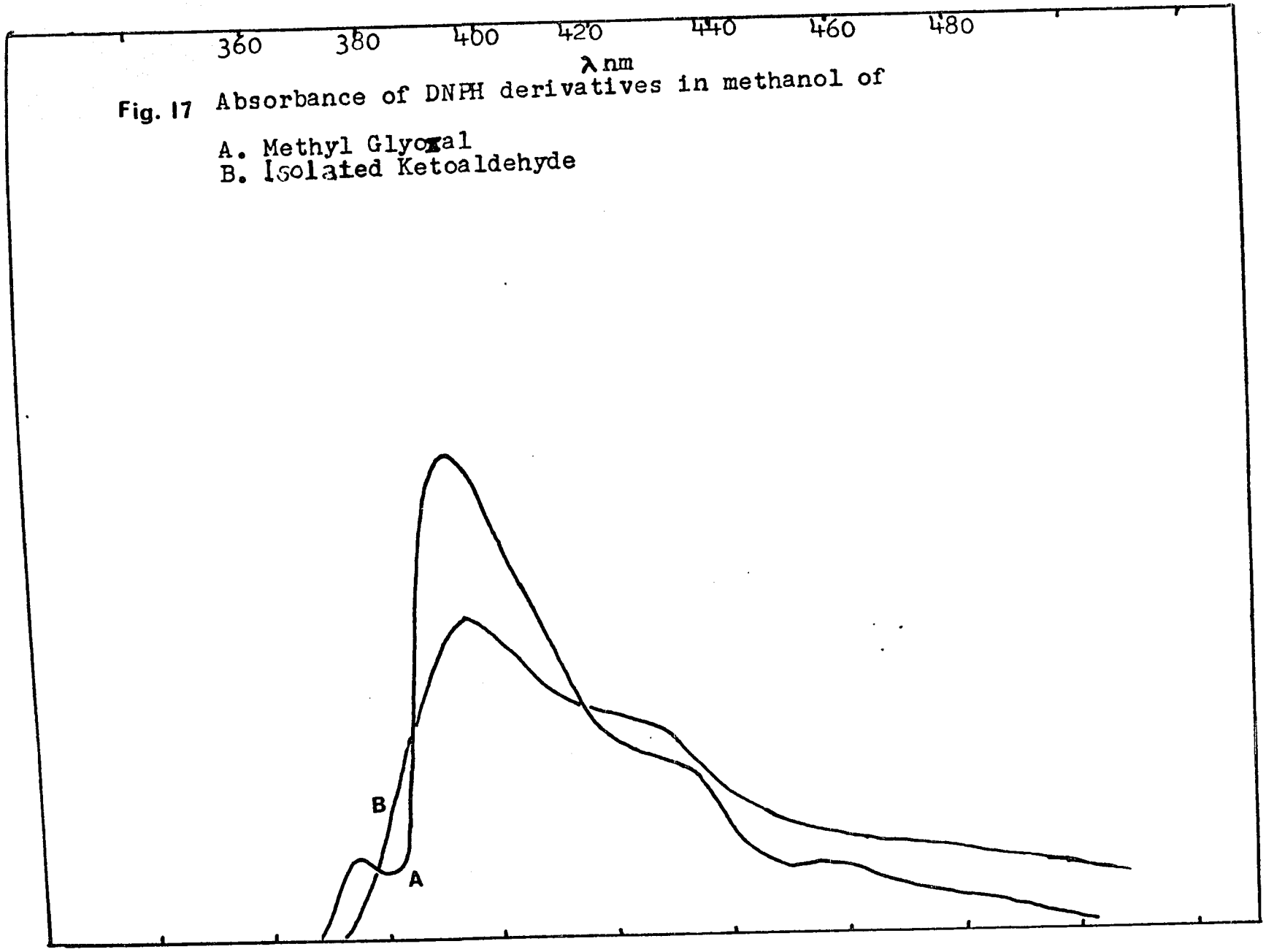


Fig. 17 Absorbance of DNPH derivatives in methanol of

A. Methyl Glyoxal
B. Isolated Ketoaldehyde

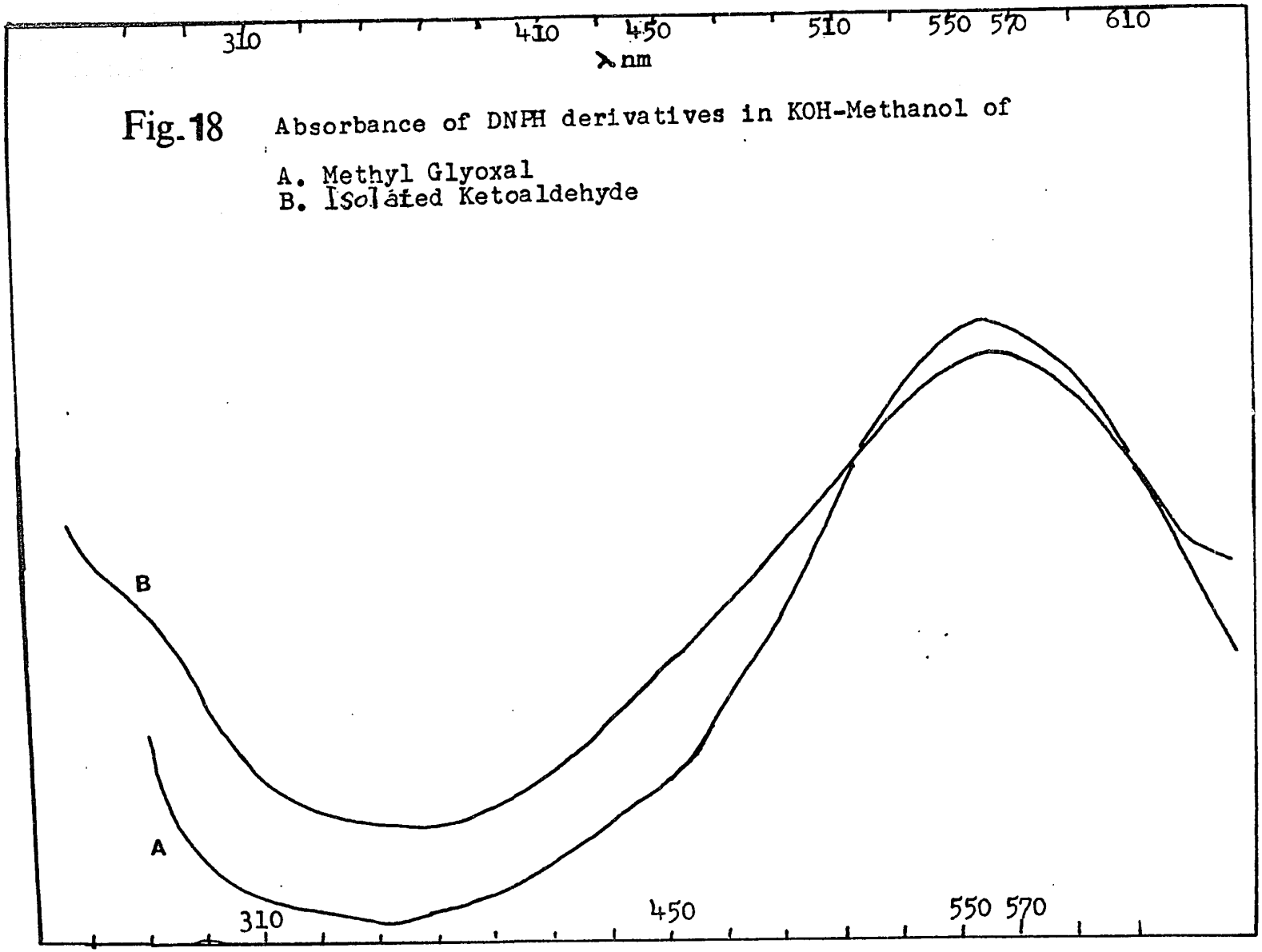


Fig. 18 Absorbance of DNPH derivatives in KOH-Methanol of

A. Methyl Glyoxal
B. Isolated Ketoaldehyde

(29) Infrared Spectroscopy of Red Crystals

Purpose:

To obtain and interpret the infrared spectrum of the dried dinitrophenylhydrazone.

Experimental:

Dry KBr (200 mg) was ground completely to a fine powder with about 13 mg of the dry red crystals. Fusion at room temperature under vacuum and 10 tons pressure produced a transparent solid disc which was placed in a Beckman IR 5 Infrared Spectrophotometer. The absorption curve was recorded.

Results: Fig. 19.

Conclusion:

(See Discussion for complete analysis of all absorption bands of the derivative). The parent molecule does not have the following groups.

Alcohol OH at 3400 cm^{-1}

Acid COOH 1700 "

Primary amine 3300 "

Ester 1735 "

Aliphatic hydrocarbon C-H stretch at & below 3000 cm^{-1} and C-H deform. from 1480 to 1430 cm^{-1} (CH_2CH_3)

There is no absence of absorptions in the 10 to 12 micron region therefore no furan (262). Absence of C-C (1020 cm^{-1}) confirms this (157).

Aromatic: 4 strong bands in the region 769 - 660 cm^{-1}.

The bands which are present, besides those belonging to the dinitrophenylhydrazine grouping, are:

A C=C stretch band at 1645 cm^{-1} the range of vinyl group (90) $HC=CH_2$

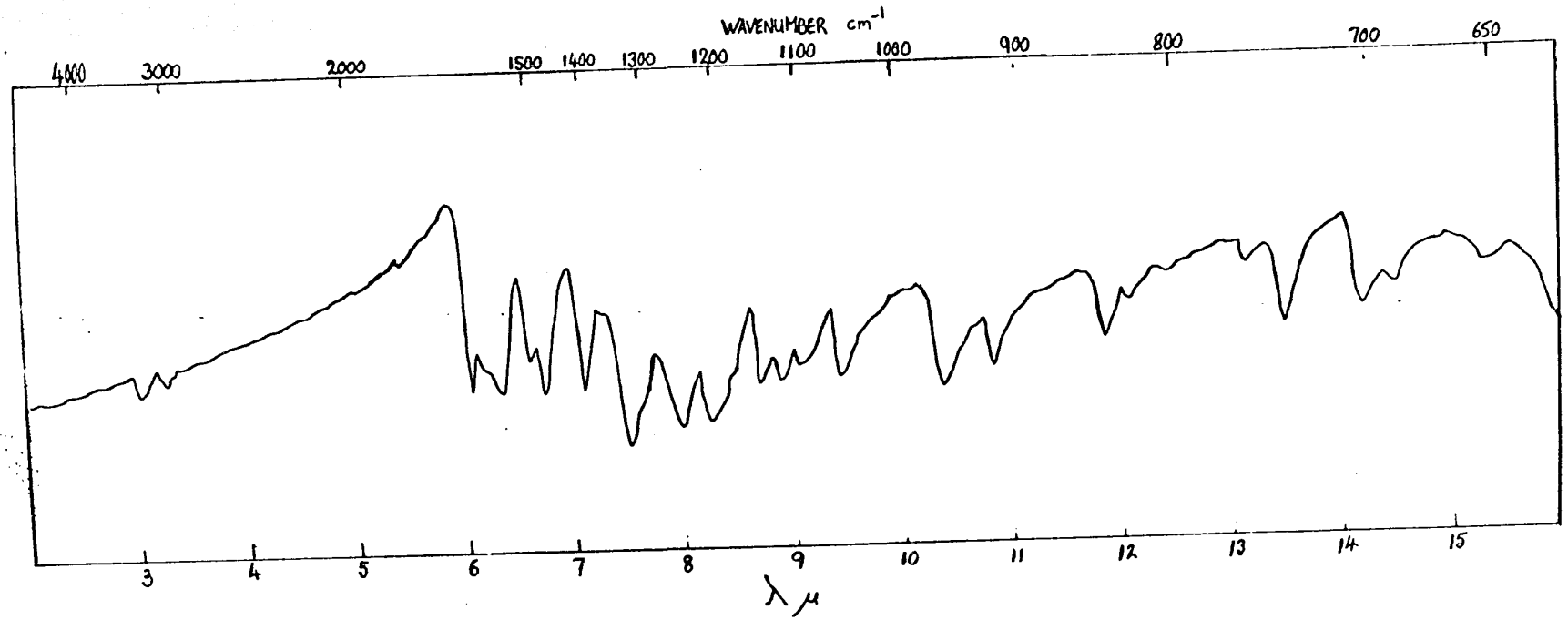


Fig. 19. I R Spectrum of the red osazone of the Ketoaldehyde from HeLa cells, in KBr disks.

absorption. Confirmation of this is seen at 1410 cm^{-1} where the in-plane deformation of $=CH_2$ absorbs. The out-of-plane bending vibrations at 962 cm^{-1} (for -CH=) and at 922 cm^{-1} (for $=CH_2$) are evident. Although other groups absorb around 920 cm^{-1} the strength of this band and the presence of its overtone at 1840 cm^{-1} indicate the vinyl group. The $_{T}CH=$ bend is usually at a higher frequency than 962 cm^{-1} but it is lowered when in the presence of electronegative groups. This would be the case if the vinyl were conjugated with the C=N derived from the keto group. The C=C stretch is strong in intensity typical of a conjugated C=C. The red colour of the derivative and the electronic spectrum indicate that the conjugation exists. The derivative of acrylaldehyde shows the same position of these latter bands not higher than 955 and 922 cm^{-1} (262) confirming that they belong to a conjugated vinyl. The derivative of acrylaldehyde displays a spectrum almost identical in this respect to the one produced here in Fig. 19.

Vinyl glyoxal is therefore considered to be the parent of the DNPH derivative in this sample.

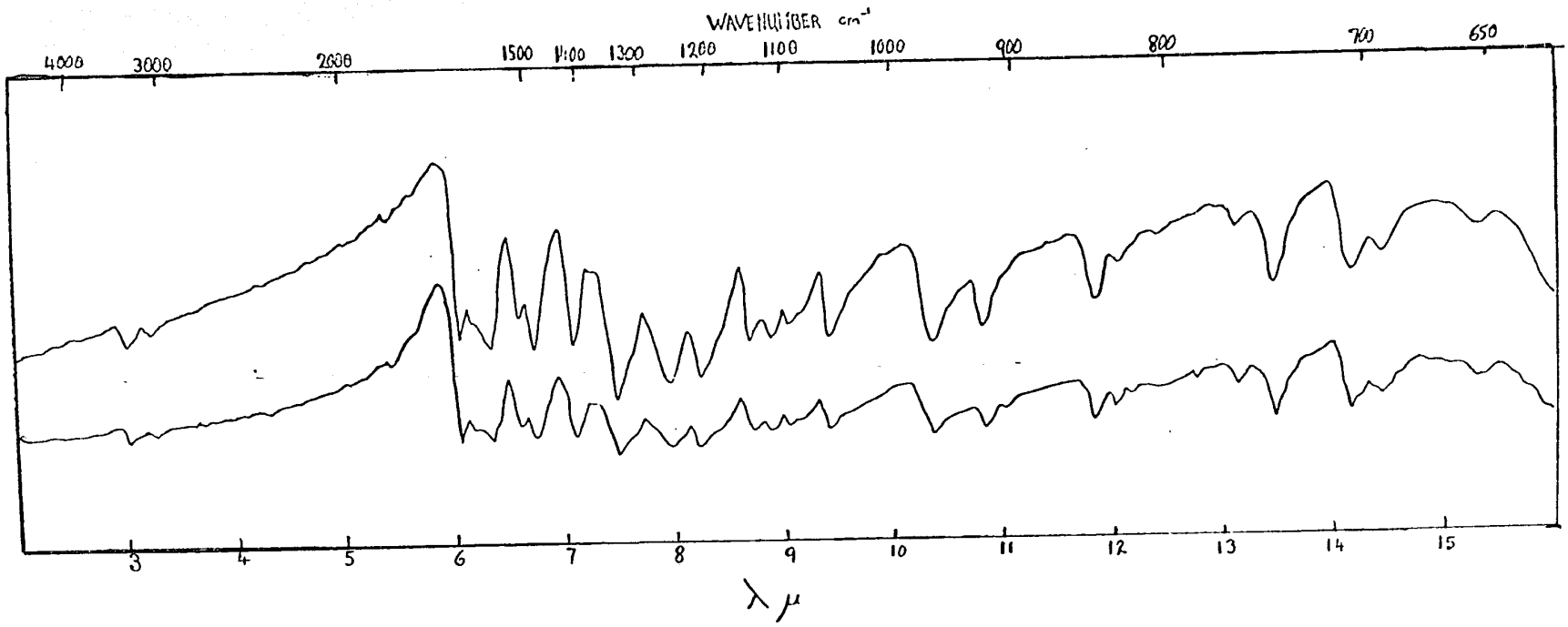


Fig. 20. I R Spectrum of the red osazone of the Ketoaldehydes from HeLa cells (Upper) and Human Kidney cells (Lower tracing), in KBr disks.

(30) Inhibitor from Human Kidney Cells

Purpose:

To attempt to isolate a DNPH derivative of a ketoaldehyde from actively inhibitory extracts of medium used by Human Kidney Cells in contrast to the HeLa cells which are used for the rest of this work.

Experimental:

Human kidney epithelial cells were shown, amongst others, to produce an inhibitor of bacterial growth. Methanol-As_2O_3 extracts were obtained from used medium fractionated on columns. They were evaporated by heating while acidic. A DNPH derivative was made. The melting point and the infrared spectrum were recorded.

Results: Fig. 20.

Melting point: 221°C.

Conclusion:

The derivative from Human Kidney Cells appears to be identical with the HeLa cell product.

(31) A Second Method of Isolation of DNPH Derivatives from HeLa cells.

Purpose:

To isolate the DNPH derivative from alcohol extracts which had not been heated.

Experimental:

As a precaution against loss of the aldehyde during heating and evaporation of the methanol and ethanol, HCl had been added in Exp. 23. This isolation procedure was changed in the present experiment. Nitrogen gas was bubbled through the methanol-As_2O_3 extract. Consequently the temperature became much lower than room temperature. To the residue ethanol was added; after 4 hours at 4°C the soluble part was removed from insoluble material. The ethanol soluble material was dried again by bubbling N_2 through it. The residue was taken to make a DNPH derivative in the cold as in experiment 26. The crystals were extensively washed and a sample chromatographed on thin layer plate. The dried crystals were studied by infrared absorption, as in experiment 29. Methyl glyoxal bis (dinitrophenylhydrazone) was made and was compared.

Results:

The derivative crystals of the ketoaldehyde from HeLa cells were yellow-orange, chromatographed as one spot, melted at 245°C. Their infrared spectrum is recorded in Fig. 21 compared to the I.R. spectrum of the methyl glyoxal osazone. The electronic spectrum is seen in Fig.22.

Conclusion:

(See Discussion, Table 26, for a complete analysis of all absorption

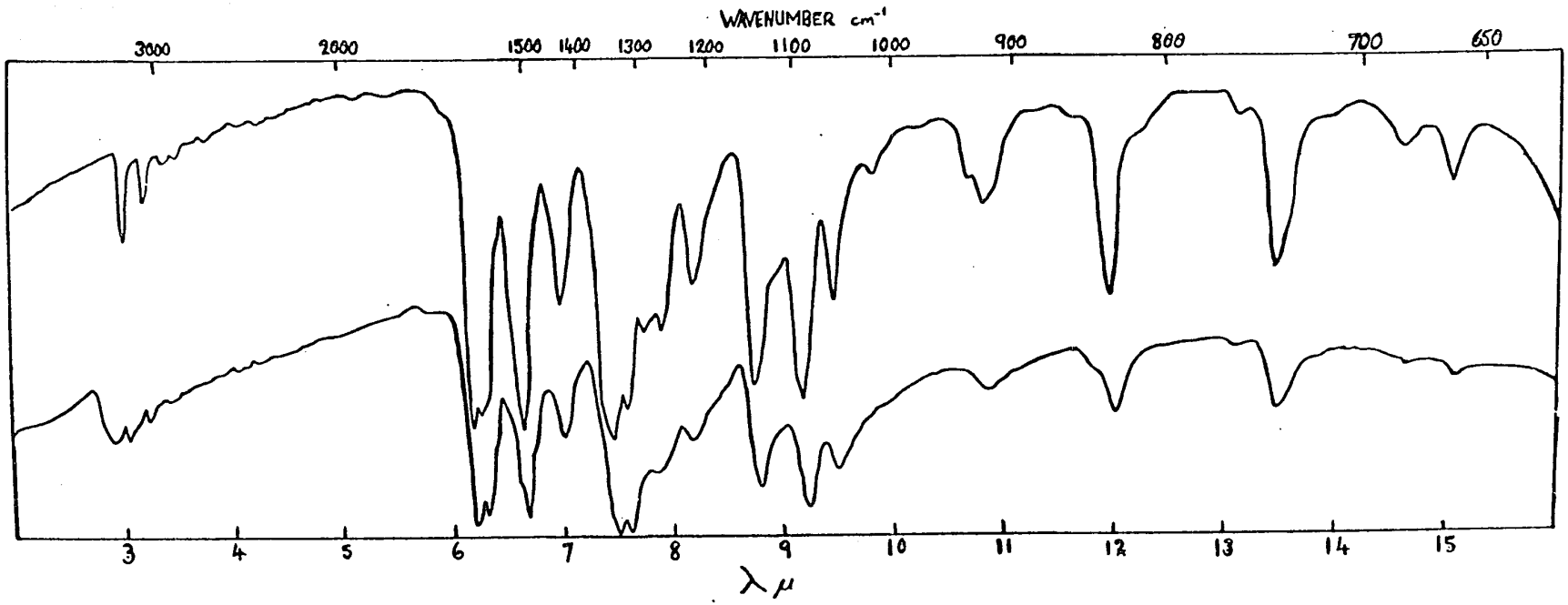


Fig. 21. I R Spectra of the yellow osazone of the Ketoaldehyde from HeLa cells (Lower) and of Methyl Glyoxal (Upper tracing), in KBr disks.

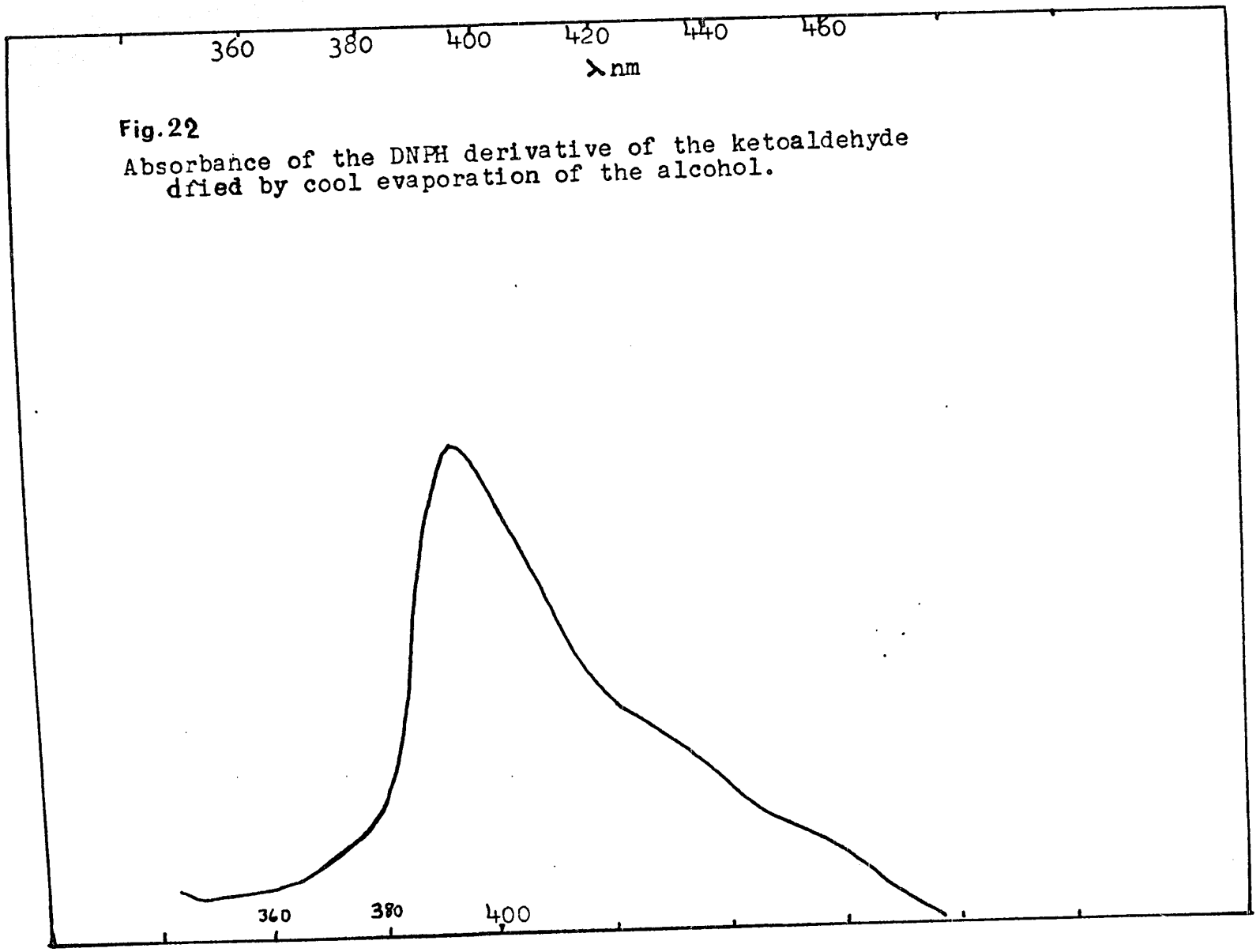


Fig.22

Absorbance of the DNPH derivative of the ketoaldehyde dfied by cool evaporation of the alcohol.

bands of the derivative). The absence of red colour would indicate a loss of the conjugation of a vinyl group with a C=N group. The spectrum shows the absence of a C=C stretch band at 1645 cm^{-1} and the absence of the -CH= deformation at 960 cm^{-1}. There remains only a weak absorption around 922 cm^{-1} of groups other than the $=CH_2$ and no overtone at 1840 cm^{-1} is visible. The $=CH_2$ in-plane deformation is missing and a band at 1420 cm^{-1} is found. It can be assigned to methylene $-CH_2-$ group in an electronegative environment, especially one holding a hydroxyl group $-CH_2OH$ (217). There appears to be a shoulder peak at 1470 cm^{-1} accounting for $-CH_2-$ aliphatic group. The presence of C-H stretch bands at 2860 cm^{-1} confirms the presence of aliphatic C-H. It is likely that no $-CH_3$ is present for there is no peak from 1430 to 1470 cm^{-1}.

The absence of the vinyl group is compensated for by the appearance of a hydroxyl group around 3450 cm^{-1}, a weak C-O stretch is discernible as a shoulder at 1036 cm^{-1}. The difference between vinyl glyoxal and this parent compound is the presence of CH_2 and OH in place of the vinyl group in the latter. In all likelihood it is 4-hydroxy-2-ketobutyraldehyde, with the hydroxyl group in a position β to the ketone. Heating such a compound in the presence of acid, as in Experiment 21, would readily dehydrate the molecule by Beta Elimination (230) of the hydroxyl group producing vinyl glyoxal as in the first compound. 4-hydroxy-2-ketobutyraldehyde is therefore believed to be the natural product. The electronic spectral information only corroborates the absence of the vinyl group. A saturated derivative is deduced from the greater absorption in the spectrum below 380 nm.

(32) Confirmation of the Dehydration due to Heating in Acid.

Purpose:

To demonstrate that heating the acidic alcohol extracts produces the dehydrated ketoaldehyde while cold evaporation prevents this in the same sample.

Experimental:

Since Experiment 26 which gave red derivative crystals, and Experiment 30 which gave yellow ones used separate lots of starting material from 2 different cultures of HeLa cells, it was necessary to confirm that the two evaporation treatments were responsible for the 2 colours and not the starting materials themselves. From one suitable column fraction 3 mls of a methanol-As_2O_3 extract were derived. Half was heated with 0.1 ml of 0.1 N HCl in a water bath to reduce the volume. The other half was bubbled with N_2 at room temperature. The former was designated 'Heated' and the latter 'Cooled'. The faint yellow colour normally seen in these extracts became stronger in intensity during heating of the 'Heated'. This can be explained as dehydration of the β hydroxy ketone producing the unsaturated conjugated vinyl form (See Discussion). To the residual $\frac{1}{2}$ ml of brown liquid 1 ml of ethanol was added. When about $\frac{1}{2}$ ml remained in the 'cooled' tube 1 ml of ethanol was added to it also and both tubes were placed on ice. Some crystalline material settled out of the 'Heated' and gelatinous matter settled out of the 'Cooled' but the volumes of each sediment appeared similar after centrifugation.

The ethanolic supernatants were removed and evaporated appropriately. 'Heated' was dried in a hot water bath and 'Cooled' was dried by a N_2 stream. When the volumes were at 0.1 ice cold absolute ethanol was added

to each ($\frac{1}{2}$ ml) and the soluble material was separated from the insoluble. A DNPH derivative was made by adding 0.1 ml of 1 N HCl and 0.3 ml DNPH reagent to the ethanol soluble material on ice. Crystals were thoroughly washed, dried and compared by electronic and I.R. spectroscopy.

Results:

After addition of the DNPH the 'Heated' was observed to form red crystals in a few minutes. From the 'Cooled' there came orange crystals. The infrared spectrum, Fig. 23 and electronic absorbance, Fig. 24, show that the two forms of crystals are identical to the compounds in experiments 24 and 31, as depicted in Figs. 19 and 21.

Conclusion:

It is evaporation by heating which produces the red derivative of vinylglyoxal and bubbling N_2 at room temperature which preserves β hydroxy ketone intact. This satisfactorily explains how both dehydrated and hydrated forms can be derived from the same sample.

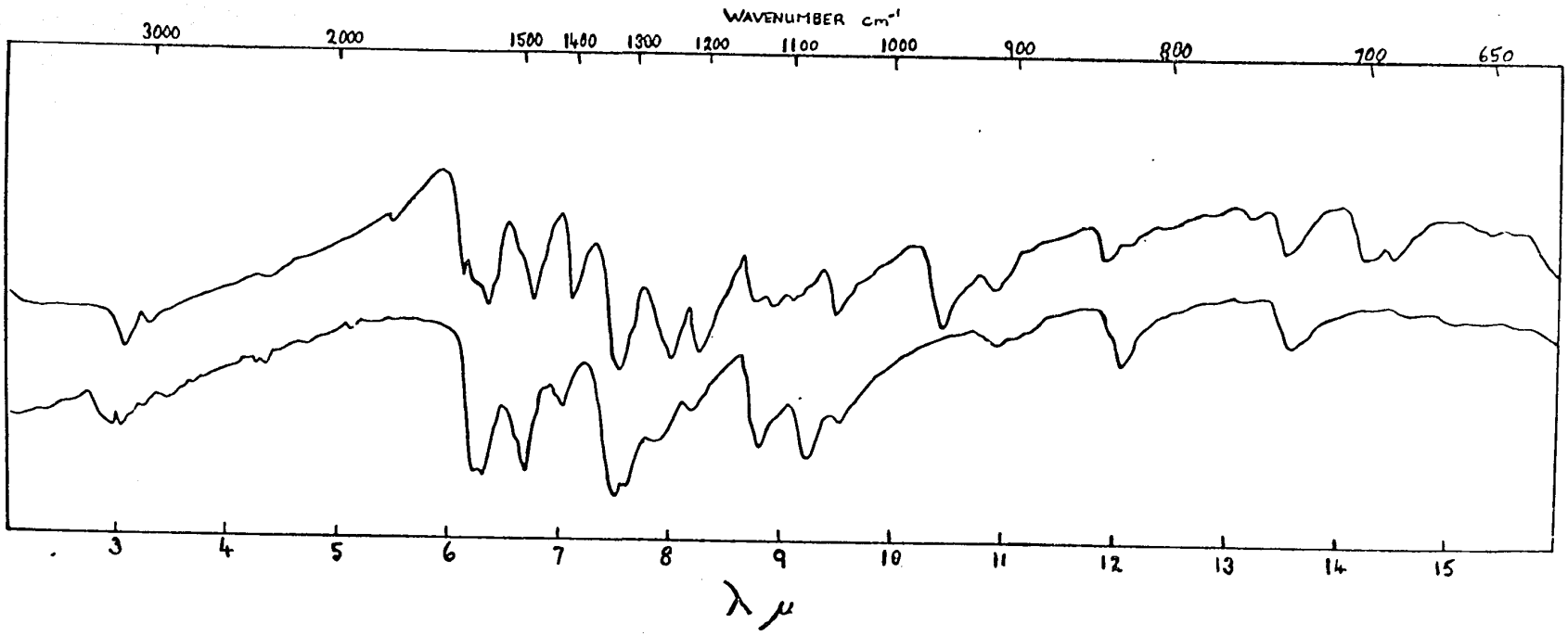


Fig. 23. I R Spectra of the red osazone from a 'heated' preparation (Upper) and the yellow osazone from a'cooled' preparation, in KBr disks.

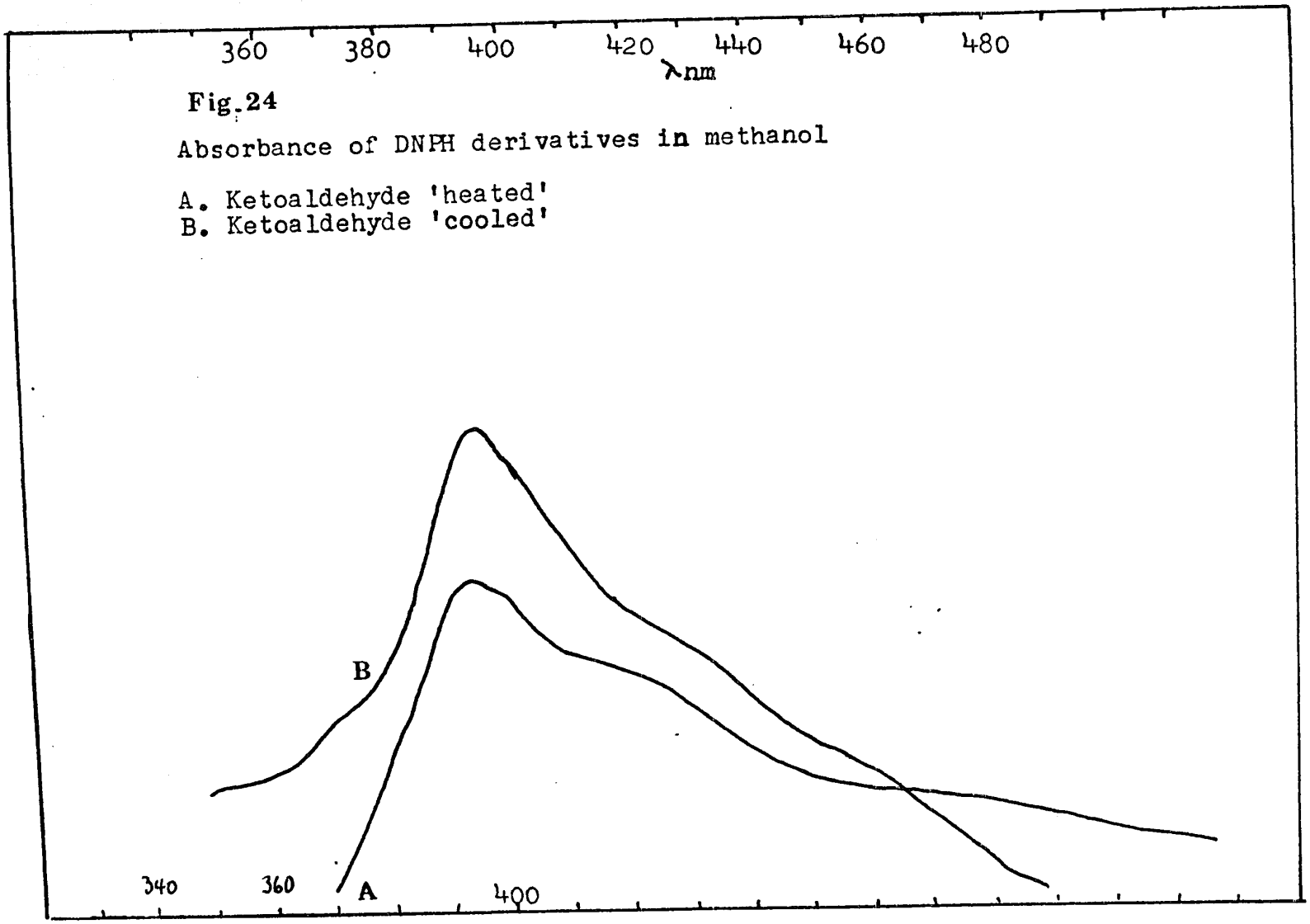


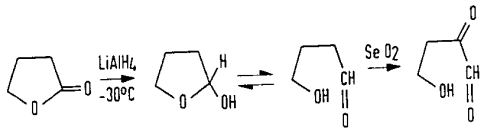
Fig.24

Absorbance of DNPH derivatives in methanol

A. Ketoaldehyde 'heated'
B. Ketoaldehyde 'cooled'

R A T I O N A L E

The rigorous interpretation of the I.R. spectra as proof of structure, although convincing, would be more acceptable if comparisons were made with a synthetic product. Synthesis of 4-hydroxy, 2-ketobutyraldehyde would also lead to the required proof sooner than repetitive osazone formation and subjection to more diverse analytical methods. An expert in synthesizing ketoaldehydes was therefore consulted. Dr. G. Fodor of Laval University suggested the two step reaction that follows.



(33) Proof of Structure by Synthesis

Purpose:

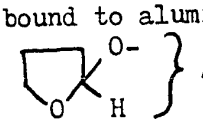
To synthesize 4-hydroxybutyraldehyde from γ butyrolactone.

Experimental:

According to Arth (18) $LiAlH_4$ can reduce a lactone to a hydroxy aldehyde (hemiacetal) if the temperature is kept below -30°C and if the hemiacetal is in a form resistant to further reduction. Higher temperature would permit complete reduction to a diol, also the presence of moisture would destroy the $LiAlH_4$. Therefore caution was taken to carry out the reaction in a room held at -20°C and in a dry-ice ethanol bath kept below -30°C. Dry glassware was taken immediately from the hot oven to the room at -20°C to keep off condensation. The solvent (THF) tetrahydrofuran, was redistilled from sodium metal to remove any water. $LiAlH_4$ was handled in the -20°C room before introduction of the frozen CO_2. Lumps were crushed cautiously to as fine a dust as possible and 2.2 gm were weighed and dissolved in 100 ml THF (in excess of 1.9 gm which is 0.05 moles) because $LiAlH_4$ is not completely soluble in THF due inevitably to some oxidation products (58) or CO_2 products. In ether the hydride would react slowly with atmospheric oxygen. Two tenths of a mole of butyrolactone (17.2 gm) (density 1.13) were quickly weighed in a small dry beaker and dissolved in 120 ml of THF and placed in a 500 ml 3 neck flask with a magnetic stirring bar. A thermometer was secured in the first neck, a tube-container of drying pellets ($CaCl_2$ or KOH) was inserted in the second and a stoppered delivery funnel was put in the third. The THF containing $LiAlH_4$ was put in the funnel and the flask was altogether held in a dry-ice ethanol bath in a plastic container

over a Magnetic stirrer. The magnet did not work through a metal container necessitating the use of a plastic one.

Slowly the $LiAlH_4$ was added dropwise to the lactone for 45 min. The temperature was constantly observed inside the flask and in the bath to assure the flask contents did not rise above -35°C. Dry-ice was supplied as needed. Stirring was seen to come to a stop when the contents became too thick; more THF at -35°C was then added. After the complete addition of the hydride stirring was allowed to continue for 1 hour as the reaction was allowed to attain, slowly, a room temperature of + 25°C.

The grayish-white paste was vacuum filtered on a sintered glass funnel until the slurry was dry. Any unreacted lactone was taken out of the slurry by repeated washings with THF. The hydroxyaldehyde was still in a form bound to aluminum in the solid phase as suggested in the formula  $AlLi^{+4}$ (18). The remaining paste in THF was added to about 50 gm ice and 3 ml concentrated sulfuric acid to dissociate the hemiacetal from the aluminum. The mixture was filtered and from the filtrate the THF layer was removed and washed with saturated aqueous Na_2SO_4. It was dried with anhydrous Na_2SO_4 and then evaporated at 30°C under slight vacuum to remove the THF. Of the remaining liquid 1 ml weighed 1.10 gm (density of 4-OH butyraldehyde 1.092 according to Paul (241)). The clear liquid was very faintly yellow.

A dinitrophenylhydrazine derivative was made which precipitated as yellow crystals. The crystals were collected and washed. The infrared spectra of the liquid aldehyde and of the DNPH derivatives were made and the melting point was determined.

Results:

Fig. 25 shows the I.R. absorption of the free aldehyde compared with

the free ketoaldehyde (Exp. 34). Fig. 26 compares the I.R. absorption of the hydrazone to two osazones (Exp. 34). The melting point of the hydrazone was recorded near 118°C.

Conclusion:

The liquid separated from the reaction mixture is 4-hydroxy-butyraldehyde. The -OH, aldehyde, $-CH_2-$ groups in the liquid and hydrazone spectra can all be accounted for (see Discussion). The melting point of 118°C agrees with Paul (241) as does the density of 1.10.

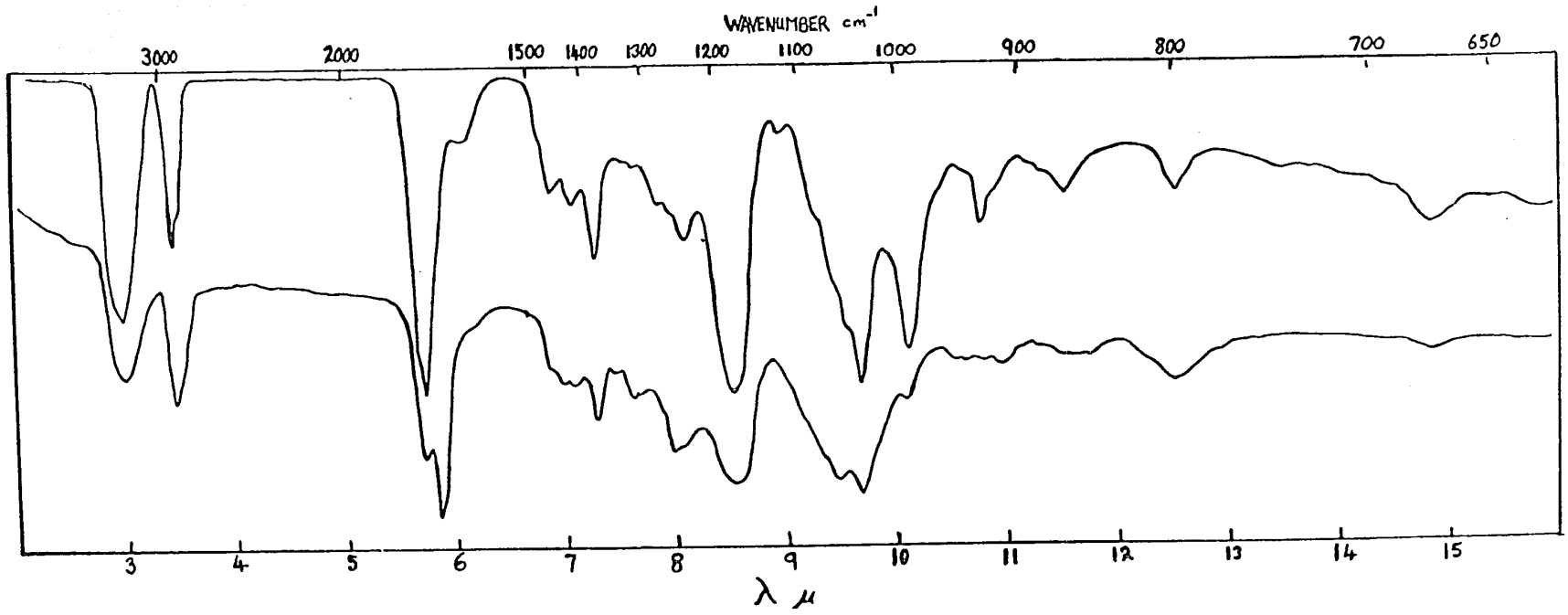


Fig. 25. I R Spectra of 4-hydroxybutyraldehyde (Upper), and of 4-hydroxy-2-ketobutyraldehyde (Lower tracing) as liquid films.

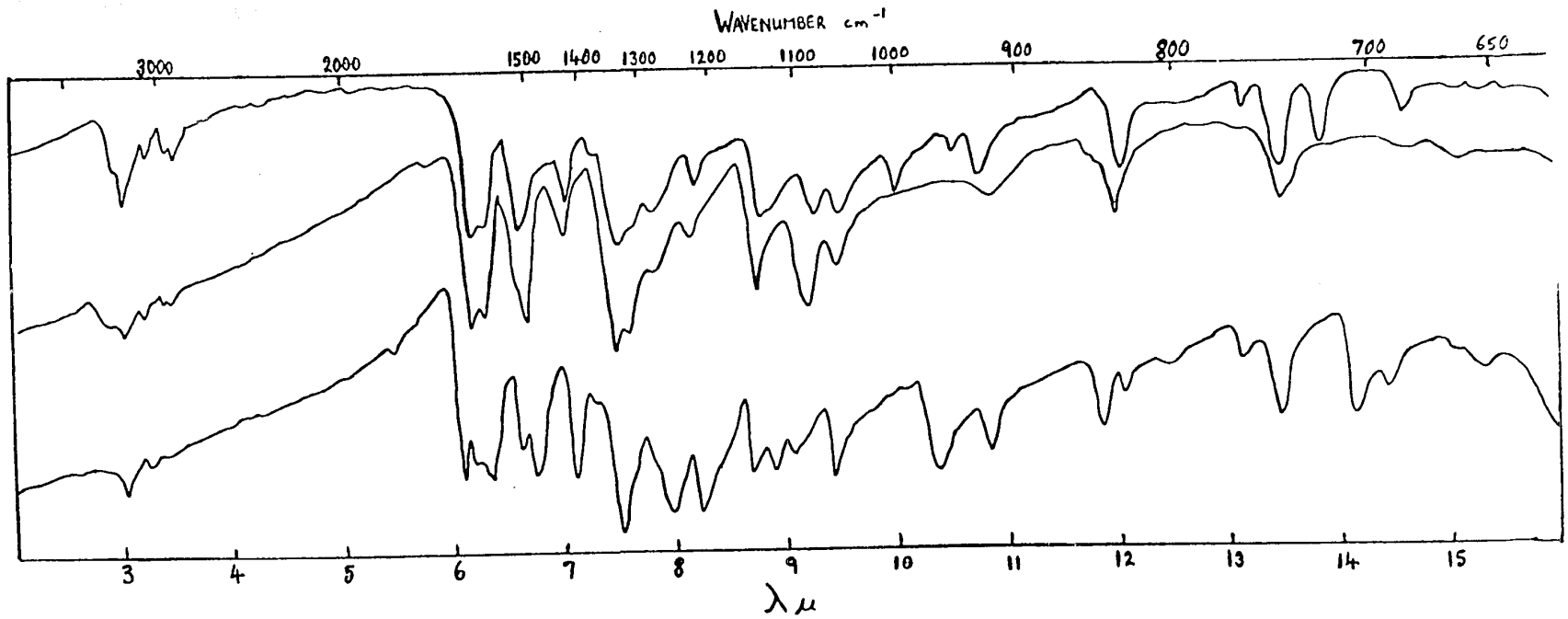


Fig. 26. I R Spectra of the hydrazone of 4-hydroxy-butyraldehyde (Upper), of the osazone of 4-hydroxy-2-ketobutyraldehyde (Middle), and of the osazone of vinyl glyoxal (Lower tracing) in KBr disks.

(34) Proof of Structure By Synthesis

Purpose:

To synthesize 4-hydroxy-2-ketobutyraldehyde from 4-hydroxybutyraldehyde.

Experimental:

One mole of SeO_2 oxidizes one mole of an aldehyde with a free α methylene group to an α -ketoaldehyde (258) (259). Therefore 1.1 gm (1/80 mole) of 4-hydroxybutyraldehyde (mw 88) was dissolved in 10 ml of 95% ethanol, and 1.387 gm (1/80 mole) of SeO_2 (mw 110.96) was dissolved separately in 40 ml ethanol. The two volumes were put together in a boiling flask fitted with a reflux condenser and heated to 80°C in a water bath. Refluxing continued for 10 hrs while black selenium precipitated and the solution turned brown.

After remaining a further 20 hrs at room temperature the ethanol solution was filtered through paper and the selenium was removed. The filtrate was then reheated under reduced pressure to remove the ethanol and any unreacted aldehyde (B.P. 65°C at 12 mm) (241). The pressure was adjusted to prevent loss of the ketoaldehyde which was seen to condense as a brown oil at the top of the flask. A little more black selenium was found to precipitate. The residual liquid was found to be dark brown and very viscous. It was dissolved in 100 ml chloroform and filtered free of selenium. The $CHCl_3$ solution was shaken vigorously with 2 N Na_2SO_4 solution to wash out water soluble selenous acid. A pinkish residue (perhaps SeO_2) was seen forming in the water phase. Washing of the chloroform solution was continued until no more selenous acid was observed in the water wash when tested for with Pb Acetate (about twelve separate washings). The chloroform solution was dried by shaking with anhydrous

Na_2SO_4, and then evaporated under reduced pressure to remove the chloroform until the volume would no longer diminish. A thick brown oil remained (0.45 ml) with the characteristic odour, sweet yet pungent, of apples rotting in an orchard, as noted in Experiment 20 for the ketoaldehyde derived from HeLa cells.

A film of liquid was made between salt blocks and the I.R. spectrum was recorded. The sample (400 µl) was dissolved in water and some was taken to make a DNPH derivative at 0°C, which turned out to be yellow orange. The washed dried crystals were examined by infrared spectroscopy, and their melting point was taken. Some of the solution was boiled in 0.01 N HCl and a DNPH derivative was made; it turned out red and was also examined by I.R. absorption.

Results:

The melting point of the yellow crystals was 246°C and of the red crystals was 221°C. The I.R. spectra of the liquid and the two osazones is seen in Fig. 25.

Conclusion:

The melting points and I.R. spectra match those of the osazones from the natural product, therefore the synthetic 4-hydroxy-2-ketobutyraldehyde may be the same compound as was isolated from the inhibitory material from HeLa cell medium. However without elemental analysis or other more rigorous methods the true identity of these compounds must remain tentative.

(35) Electronic Spectral Analysis of Ketoaldehydes

Purpose:

To examine and compare the electronic spectra of hydroxyethyl glyoxal and vinyl glyoxal, synthetic and naturally derived, to compare molecular structures and their tautomers in diverse solvent environments.

Experimental:

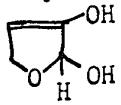
Synthetic hydroxyethyl glyoxal was dissolved in hexane, chloroform, ethanol and water and the absorption spectra were recorded (Fig. 27). In ethanol the spectrum was recorded of hydroxyethyl glyoxal with HCl and with NaOH (Fig. 28). In comparison methanol-As_2O_3 extracts of active column fractions were studied in cyclohexane, methanol, and water (Fig.29). Some synthetic hydroxyethyl glyoxal was dehydrated by heating in acidified ethanol. The absorption of the resultant vinyl glyoxal was recorded in ethanol and with NaOH (Fig. 30). Methyl glyoxal was dissolved in hexane, chloroform, ethanol and water and the absorbance was recorded (Fig. 31).

Results:

Figures 27, 28, 29, 30 and 31 are the electronic absorption spectra of the ketoaldehydes in various solvents.

Observations and Conclusions:

The spectra of hydroxyethyl glyoxal, both synthetic and natural, lack strong absorptions around 280 nm (Fig. 27 and Fig. 29).

Methyl glyoxal and vinyl glyoxal do show high absorbance in this region (Fig. 30 and Fig. 31). This observation suggests that hydroxyethyl glyoxal is in the enol and hemiacetal tautomeric form 

. Acid can open the ring and with the freeing of the aldehyde

Fig.27

U.V. Spectra of Synthetic hydroxyethyl glyoxal dissolved in
A. Hexane
B. Chloroform
C. Ethanol
D. Water

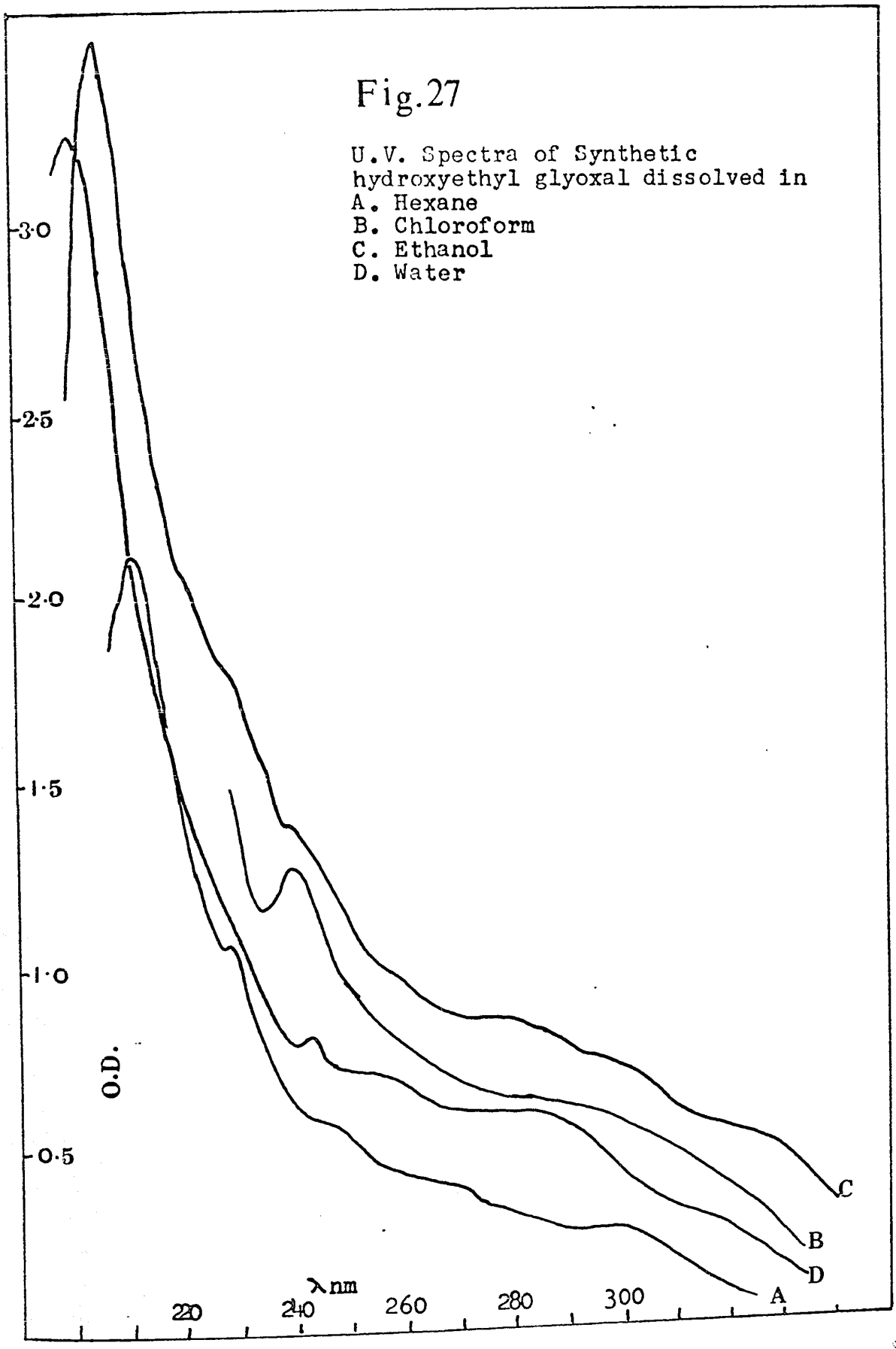


Fig.28 U.V. Spectra of hydroxyethyl glyoxal in ethanol in equal volume with

A. 0.5 N HCl
B. 0.5 N NaOH

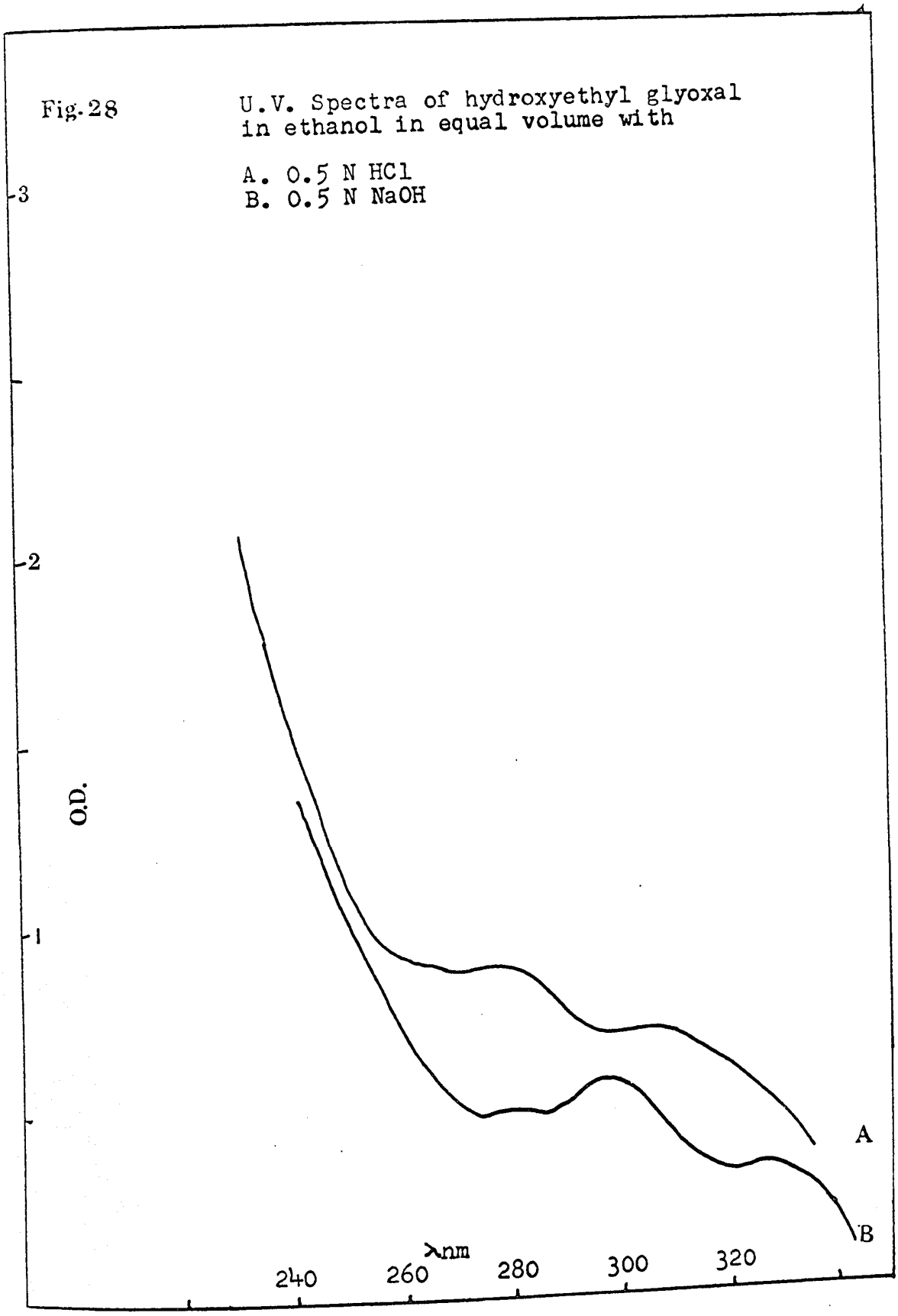


Fig.29

U.V. Spectra of concentrated Methanol-As_2O_3 extracts in

A. Cyclohexane
B. Methanol
C. Water

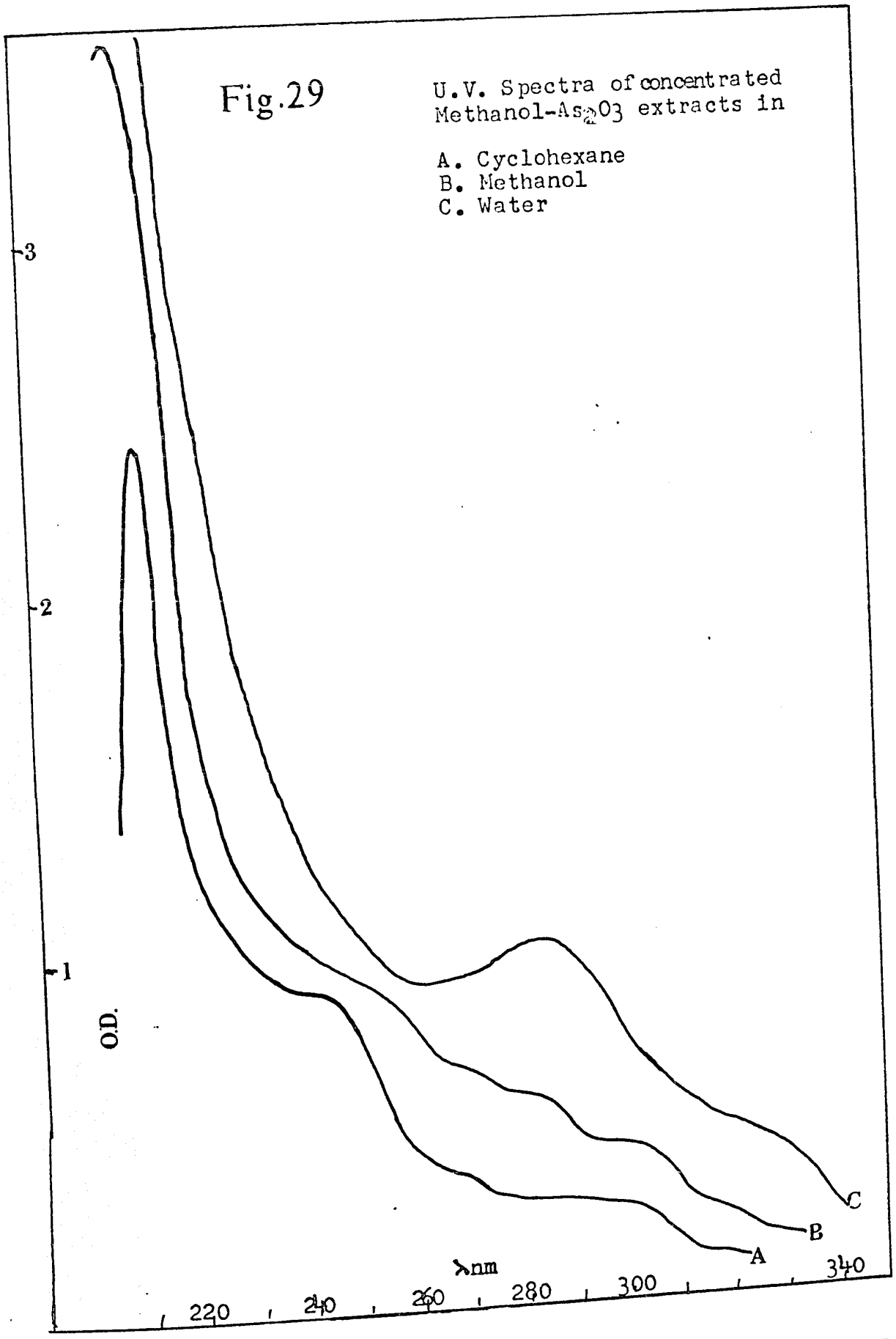
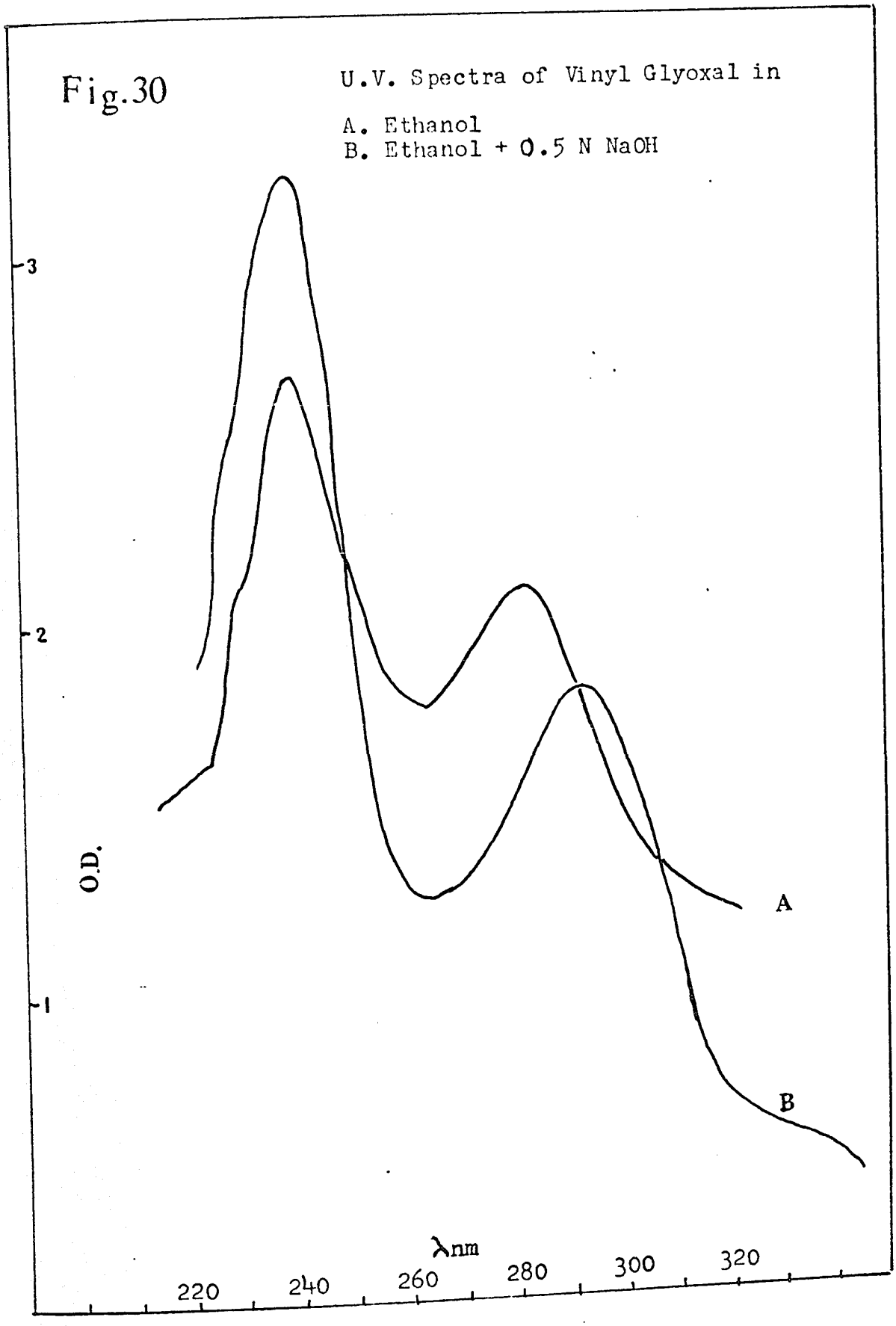


Fig.30

U.V. Spectra of Vinyl Glyoxal in

A. Ethanol
B. Ethanol + 0.5 N NaOH



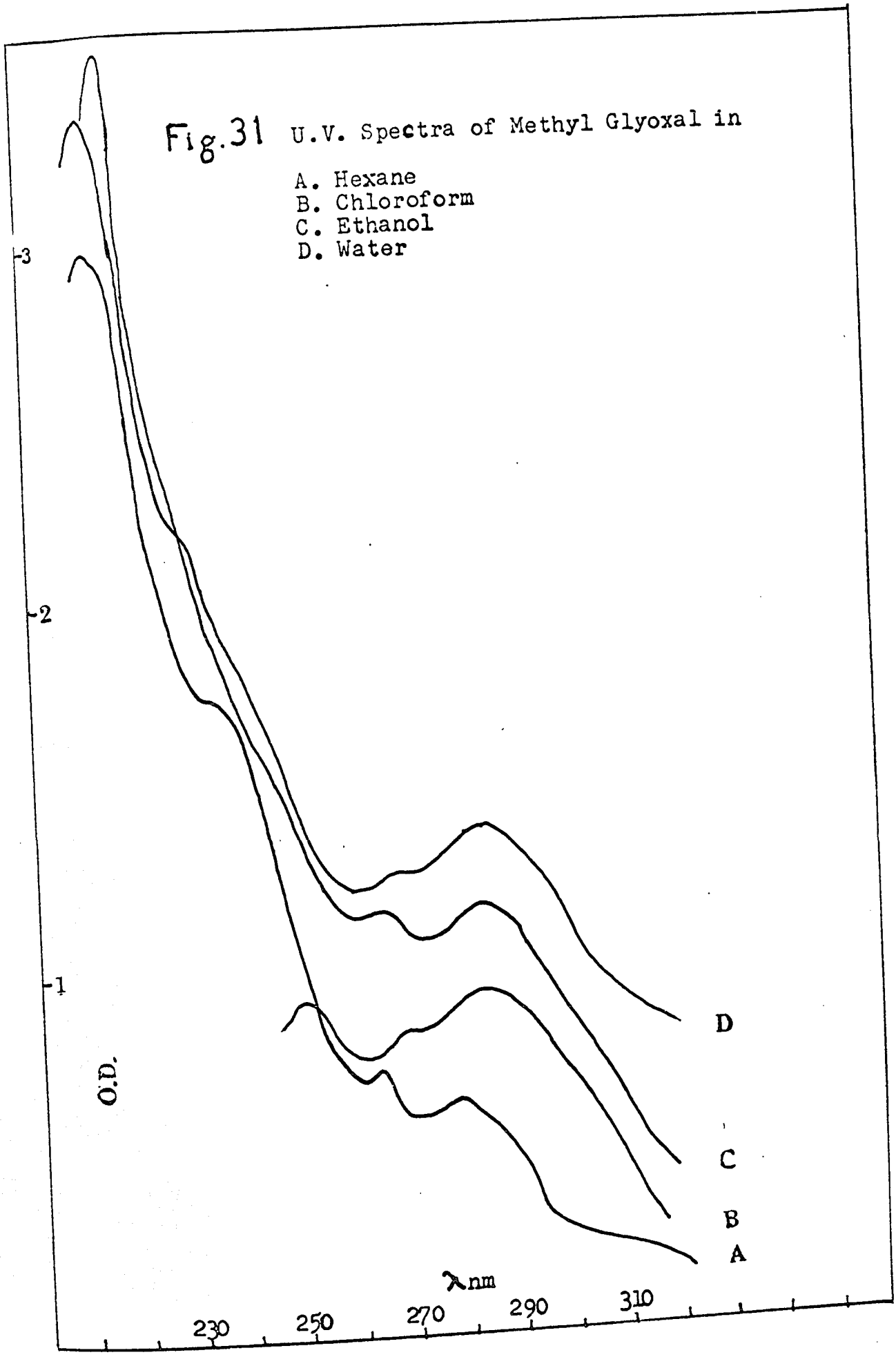
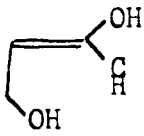
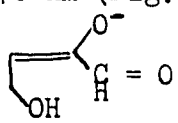


Fig.31 U.V. Spectra of Methyl Glyoxal in
A. Hexane
B. Chloroform
C. Ethanol
D. Water
3
2
1
O.D.
D
C
B
A
λnm
230
250
270
290
310

an enal  contributes to absorption at 270 nm (Fig. 28). NaOH also opens the ring but creates an enolate anion  with absorption at 292 nm (Fig. 28). This is seen for both synthetic and natural (Fig. 7) hydroxyethyl glyoxal. The similar absorbance of these and their behaviour under solvent changes, demonstrate that synthetic hydroxyethyl glyoxal and the methanol-As_2O_3 extracts may have structural identity, and exist possibly in both cyclic and acyclic arrangements depending on environmental conditions.

Vinyl glyoxal and the heated extracts display similar spectra as evidence of their identity. In particular the enone CH_2=CH-CO-CHO absorption at 240 nm, Fig. 30 and Fig. 13, is clear. A slight contribution from the allene CH_2=C=C(OH)-CH=O at 227 nm is evident and the resulting hydroxy enal absorbs around 275 nm. This allene-ol tautomer must be in small amount as NaOH does not raise or shift the absorbance by very much in making the enolate anion at 290 nm (Fig. 30 and Fig. 13). Both synthetic and natural vinyl glyoxal show their identity and in contrast to hydroxyethyl glyoxal must always be in the open form where the carbonyls are free.

Although the infrared spectrum Fig. 25 indicates the two ketoaldehyde carbonyls are free the fact that they are absent in the electronic spectrum is probably due to the fact that in solvents the tendency to form the hemiacetal is greater than in pure liquid.

F. BIOLOGICAL ACTIVITY OF KETOALDEHYDES - EXPERIMENTS 36 to 38

(36) Hydroxyethylglyoxal As a Substrate for Glyoxalase

Purpose:

To examine whether the synthetic hydroxyethyl glyoxal is a substrate for the glyoxalase enzyme.

Experimental:

Following the assay of Racker (174) Glyoxalase I enzyme from yeast (Sigma Chemicals) was dissolved in an 0.1% Bovine Serum Albumin solution in 0.01 M Phosphate buffer at pH 7.4, to give a concentration of 10 µg of enzyme protein per ml. One tenth ml of this was added to a cuvette containing substrate and coenzyme Glutathione. The O.D. at 240 nm was read every 30 seconds against a water blank, to measure the amount of thioester formed between Glutathione and the Ketoaldehyde substrate. The substrates separately used were methyl glyoxal 0.1 M (O.D. 276 nm: c 0. 620), hydroxyethyl glyoxal (O.D. 276 nm: 0.600) and dried ethanol extract (Experiment 31) (O.D. 276 nm: c 0.600). Into each cuvette was placed 2.0 ml of water, 0.1 of 1 M Phosphate buffer at pH 6.6, 0.5 ml of 0.2% glutathione in phosphate buffer at pH 6.6, 0.5 ml of the substrate solution and finally 0.1 ml of the enzyme solution. Timing began when the enzyme solution was added.

Results:

All three substrates reacted with glutathione, as seen in Fig. 32, to form a thioester absorbing light at 240 nm.

Conclusion:

Hydroxyethylglyoxal is a substrate for the glyoxalase I enzyme.

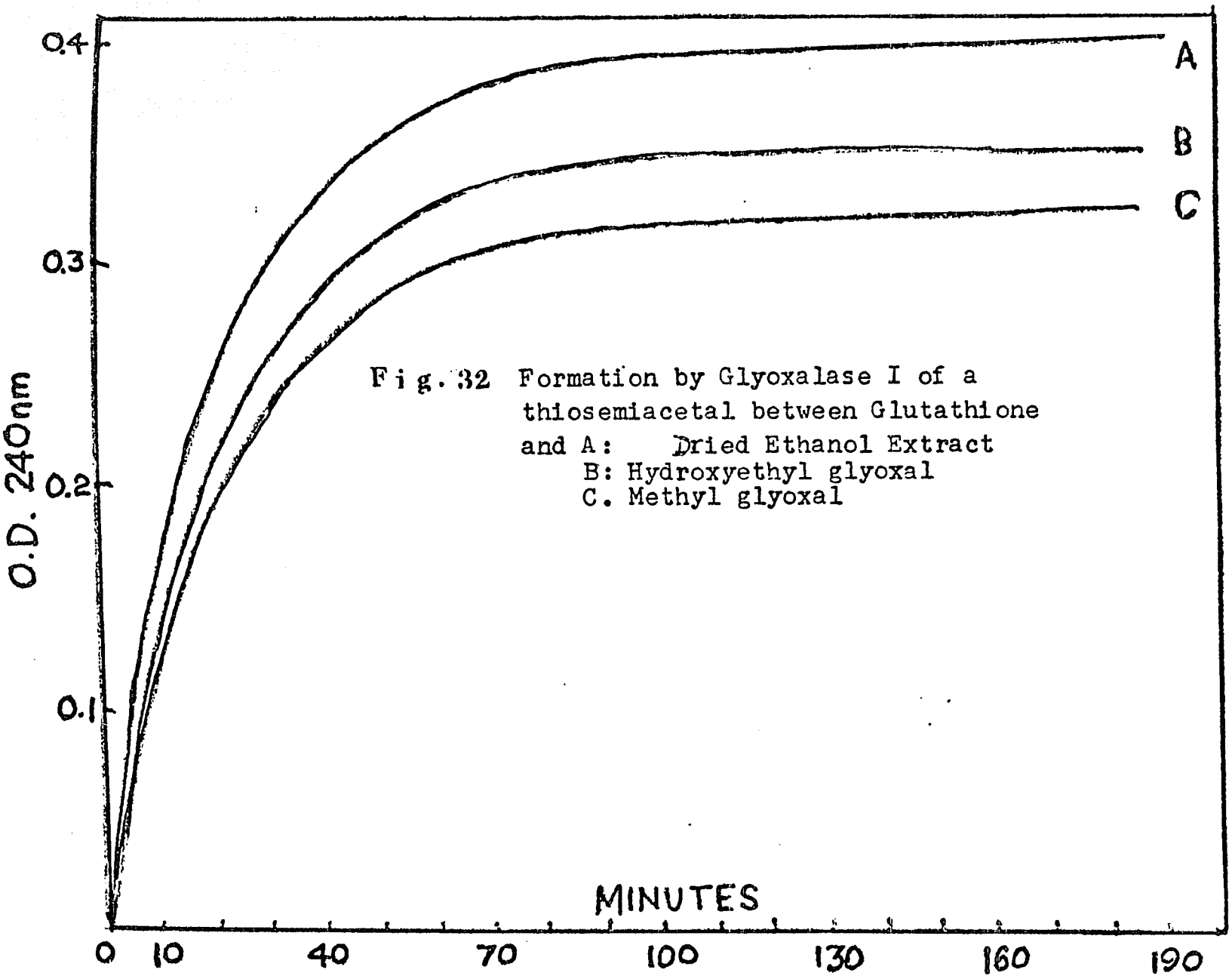


Fig. 32 Formation by Glyoxalase I of a thiosemiacetal between Glutathione and A: Dried Ethanol Extract
B: Hydroxyethyl glyoxal
C. Methyl glyoxal

(37) Assay of Synthetic Material Against Bacteria

Purpose:

To examine the synthetic 4-OH, 2-ketobutyraldehyde for activity against bacterial growth.

Experimental:

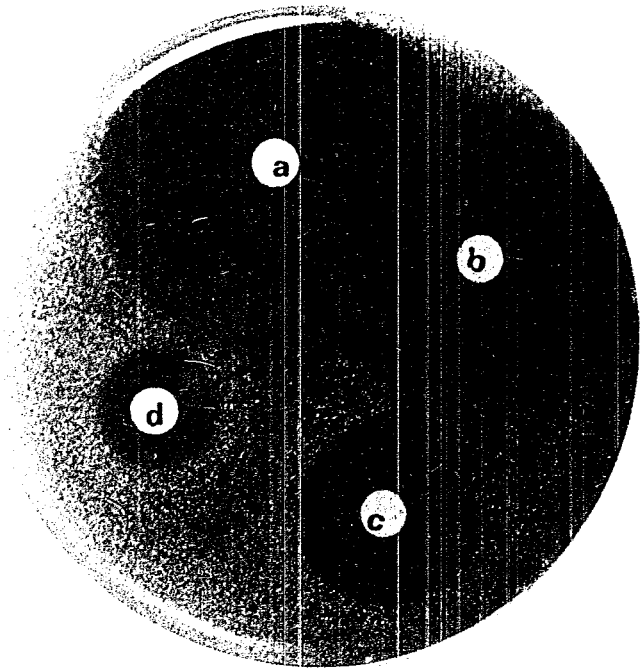
Four hundred microlitres of the ketoaldehyde weighed 0.416 gm therefore the density was 1.04 gm/ml. The molecular weight of the ketoaldehyde is 102 therefore 0.408 gm is 0.004 moles. The 400 µl (0.416 gm) was made up to 8 ml with water giving a solution of close to 0.5 M. Some of this solution was diluted with water 1 in 10 and 1 in 100 to give 5×10^{-2} and 5×10^{-3} M. The solutions were filtered sterile before use. In a like manner solutions were made to the same concentrations for a comparative study of methyl glyoxal, of Kethoxal (α keto-β -ethoxy butyraldehyde) (118), and of the natural ketoaldehyde from HeLa cells, that is the dried ethanol extract (Cold) from Experiment 31. Concentration was calculated roughly from the O.D.

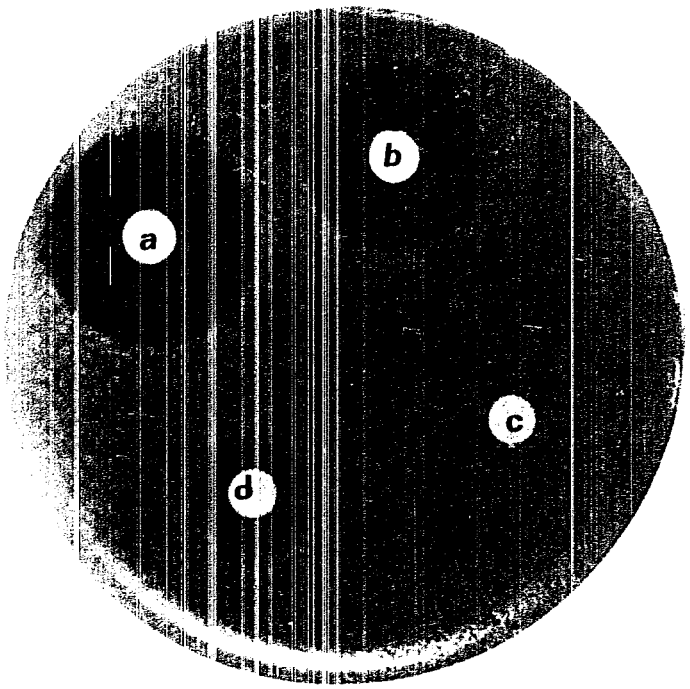
Both tube assay and disc assays were used. For the latter fifty microlitres of each solution was soaked into sterile filter paper discs (diameter 6 mm) which were then placed with sterile forceps on to agar plates seeded with the chosen bacterial strains. Growth was observed after 24 hrs.

For the tube assays 10^5 organisms were inoculated into 2 ml medium 199 containing separately the four ketoaldehydes at concentrations from 10^{-3} to 10^{-5} M. In addition γ butyrolactone was assayed as a control.

Results:

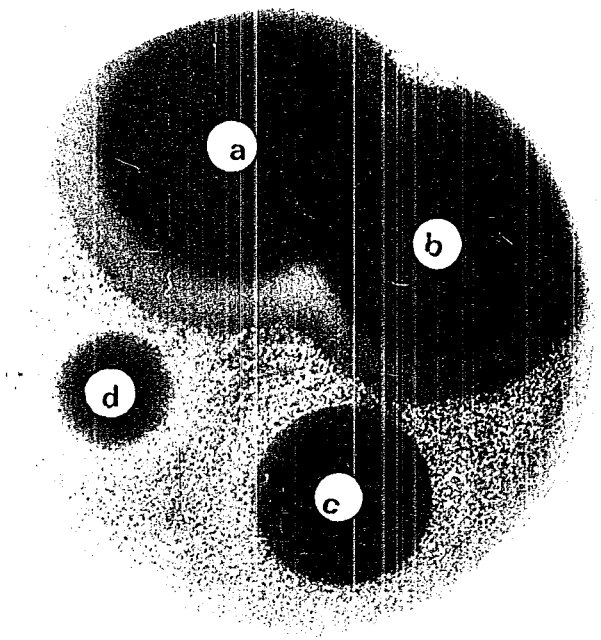
Figs. 33, 34, 35 show the disc assay against a less virulent strain of

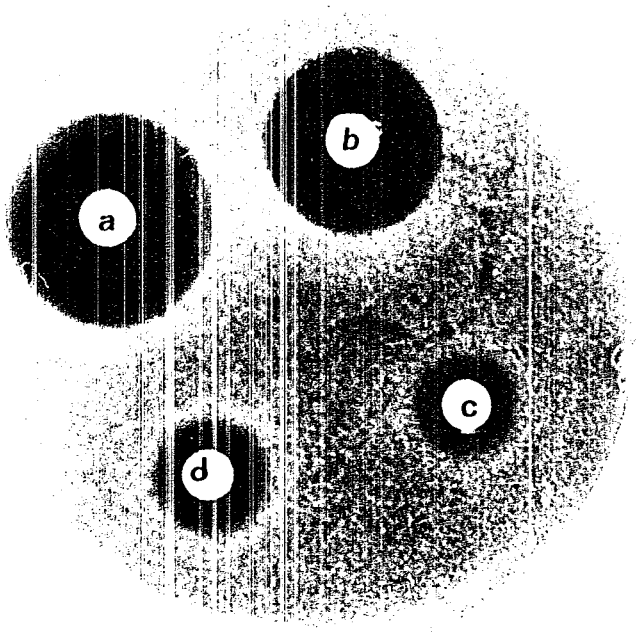




Figs. 33 and 36.

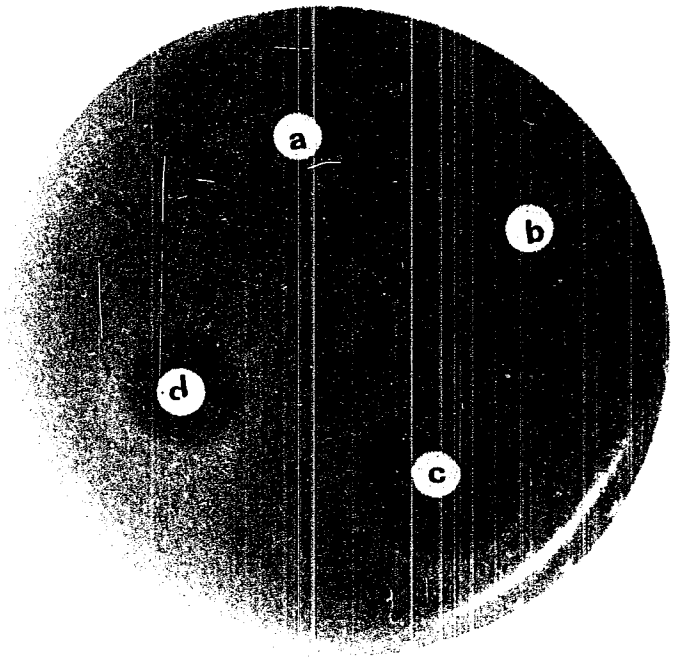
Inhibition of growth of a less virulent strain (Fig. 33) and a virulent strain (Fig. 36) of S. aureus by a: dried ethanol extract, b: the synthetic ketoaldehyde, c: methyl glyoxal and d: Kethoxal, all at a concentration of 0.5 M.

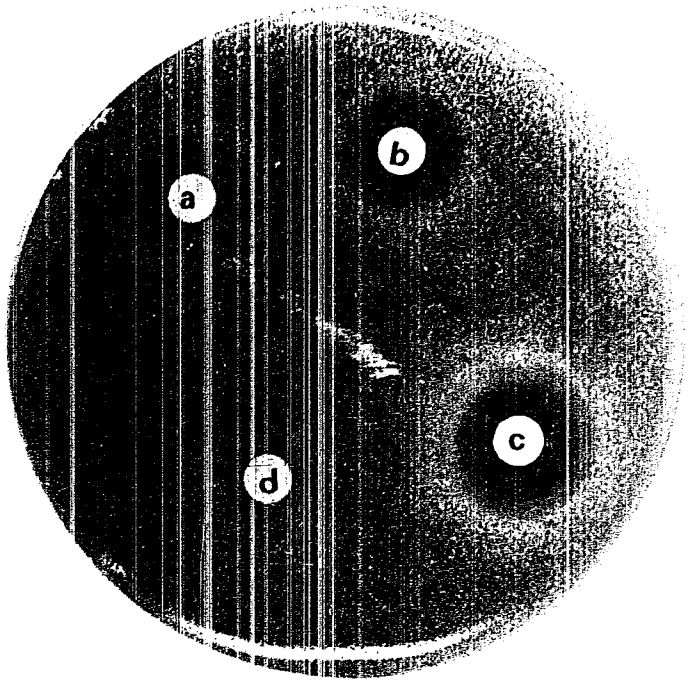




Figs. 33 and 36.

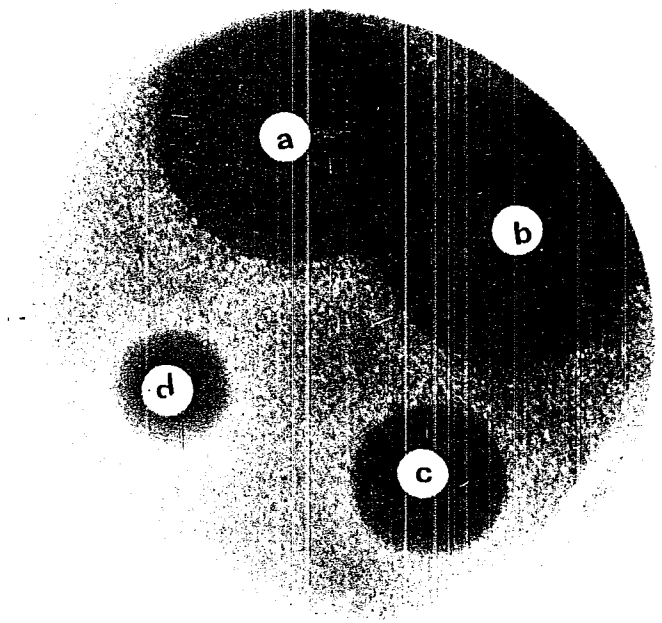
Inhibition of growth of a less virulent strain (Fig. 33) and a virulent strain (Fig. 36) of S. aureus by a: Dried ethanol extract, b: the synthetic ketoaldehyde, c: methyl glyoxal and d: Kethoxal, all at a concentration of 0.5 M.

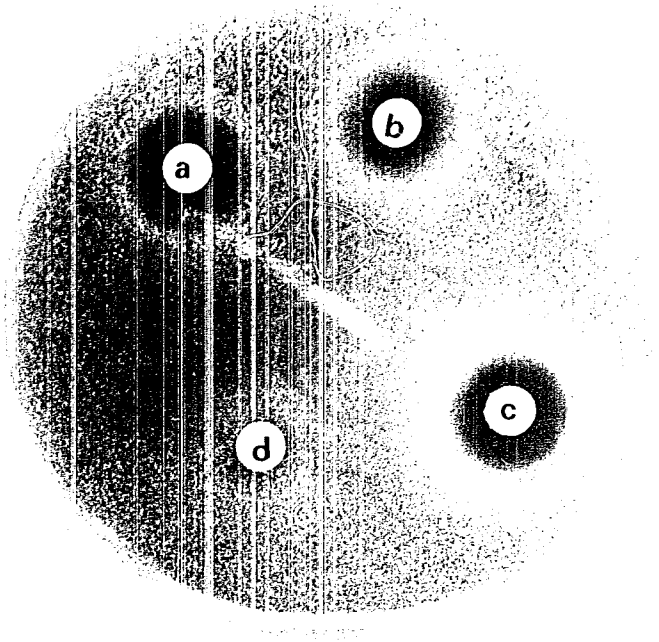




Figs. 34 and 37.

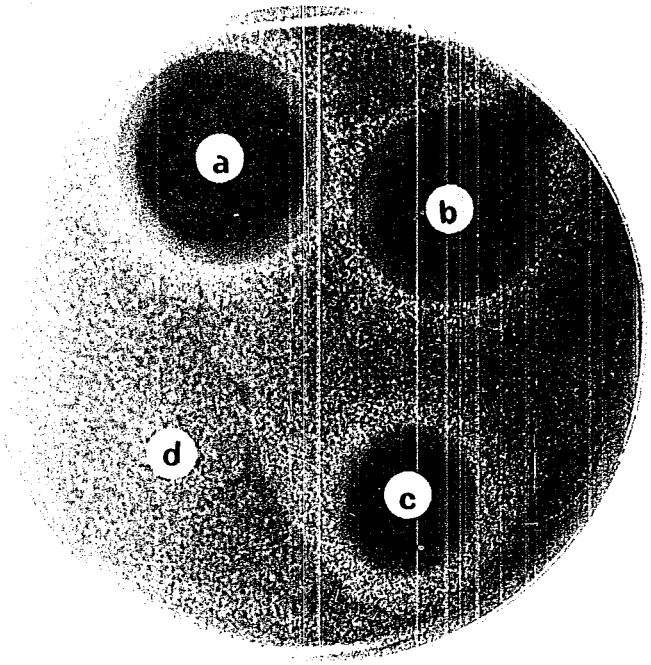
Inhibition of growth of a less virulent strain (Fig. 34) and a virulent strain (Fig. 37) of S. aureus by a: dried ethanol extract, b: the synthetic ketoaldehyde, c: methyl glyoxal and d: Kethoxal, all at a concentration of 0.05 M.

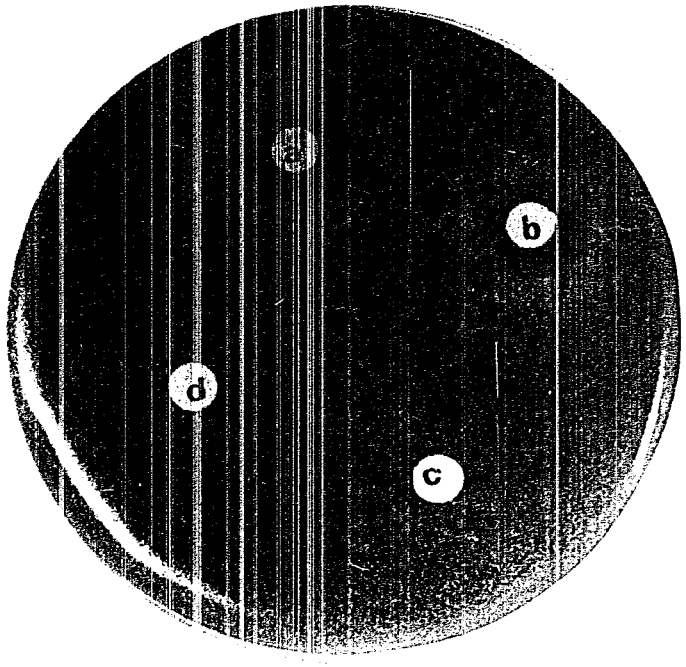




Figs. 34 and 37.

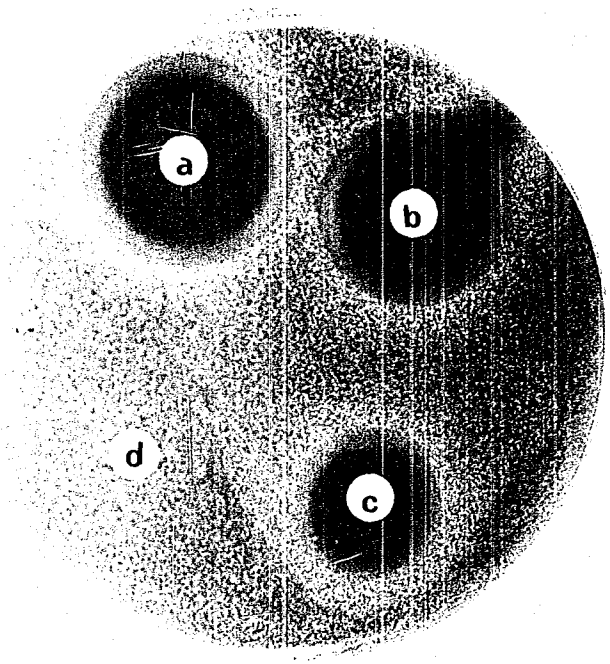
Inhibition of growth of a less virulent strain (Fig. 34) and a virulent strain (Fig. 37) of S. aureus by a: dried ethanol extract, b: the synthetic ketoaldehyde, c: methyl glyoxal and d: Kethoxal, all at a concentration of 0.05 M.

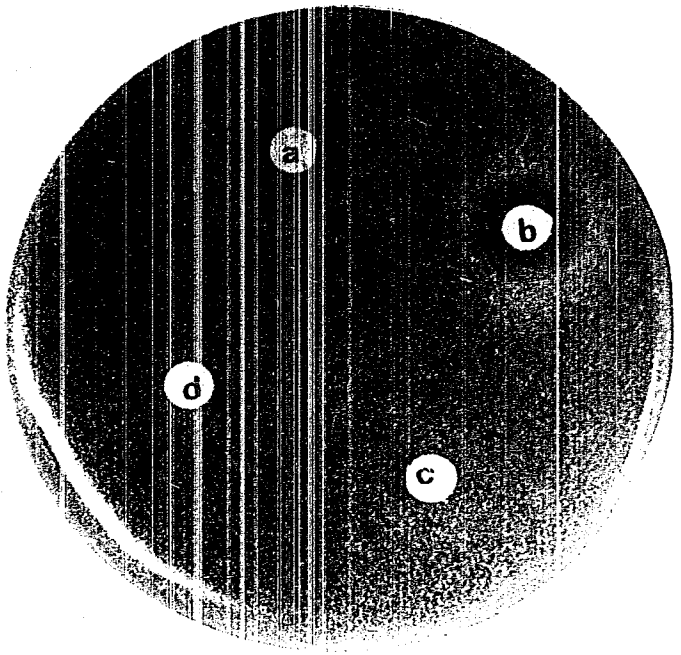




Figs. 35 and 38.

Inhibition of growth of a less virulent strain (Fig. 35) and a virulent strain (Fig. 38) of S. aureus by a: dried ethanol extract, b: the synthetic ketoaldehyde, c: methyl glyoxal and d: Kethoxal, all at a concentration of 0.005 M.





Figs. 35 and 38.

Inhibition of growth of a less virulent strain (Fig. 35) and a virulent strain (Fig. 38) of S. aureus by a: dried ethanol extract, b: the synthetic ketoaldehyde, c: methyl glyoxal and d: Kethoxal, all at a concentration of 0.005 M.

S. aureus from phage group II. The purified ethanol extract (A) and the synthesized ketoaldehyde (B) showed strong growth inhibitory activity. The 50 µl of the 0.5 M concentration discs gave zones of inhibition of about 2.8 -3.1 cm (Fig. 33). At 0.05 M zones were about 2.2 cm (Fig. 34) and at 0.005 M zones were about 1.7 cm (Fig. 35). See Table 23.

Against the more virulent strain of bacteria (Figs. 36, 37, 38) the purified ethanol extract (A) and the synthetic hydroxyethylglyoxal (B) showed less inhibition. Zones were about 1.8 cm for the 0.5 M solution (Fig. 36), about 0.8 cm at 0.05 M (Fig. 37) and just around the disc at 0.005 M (Fig. 38).

Methyl glyoxal (C in Figs. 33-38) inhibited less than the hydroxyethyl glyoxal with a zone of 1.7 cm at 0.5 M (Fig. 33), of 1.4 cm at 0.05 M (Fig. 34), and 1.1 cm at 0.005 M (Fig. 35) against the less virulent strain. Against the virulent strain methyl glyoxal shows slight inhibition at 0.5 M (Fig. 36) and 0.05 M (Fig. 37) and none at 0.005 M (Fig. 38).

Kethoxal (D in Figs. 33-38) shows inhibition least of all. None appears at 0.005 M against the virulent (Fig. 38) or less virulent(Fig.35) strains. At 0.05 M the virulent strain is not inhibited (Fig. 37) but the less virulent one is inhibited slightly (Fig. 34). At 0.5 M the zone is about 0.8 cm (Figs. 33 & 36).

Table 24 indicates growth in the Tube assays. The synthetic and natural ketoaldehydes are more active than the others. γ butyrolactone has no inhibitory activity on bacterial growth at the concentrations tried.

Conclusions:

The type of inhibition observed with 4-OH,2-ketobutyraldehyde and purified ethanol extracts is similar to that observed originally with the

TABLE 23. Sizes of Zones of Inhibition from Figs. 33 to 38.

Ketoaldehyde Concentration	Material	Zone diameters	
		Fig.33	Fig. 36
0.5 M	a	31 mm	20 mm
	b	28 "	17 "
	c	17 "	7 "
	d	8 "	8 "
		Fig.34	Fig.37
0.05 M	a	23 "	9 "
	b	22 "	8 "
	c	14 "	10 "
	d	7 "	0 "
		Fig.35	Fig.38
0.0005 M	a	19 "	8 "
	b	17 "	8 "
	c	12 "	0 "
	d	0 "	0 "

a, b, c, d, defined as in Figs. 33 to 38.

TABLE 24. Assay of Bacterial Growth with Methyl Glyoxal, Kethoxal, 4-hydroxy, 2-ketobutyraldehyde (Synthetic) and the ketoaldehyde from HeLa cells, with γ butyrolactone as a control. Observations recorded after 24 hrs; + indicates growth.

Chemical	Concentration	Bacterial Growth (S. aureus Phage group)		
		I	II	III
Methyl glyoxal	5×10^{-3} M	+	±	±
	5×10^{-4} M	2+	±	2+
	5×10^{-5} M	2+	2+	2+
Kethoxal	5×10^{-3} M	3+	3+	3+
	5×10^{-4} M	4+	4+	4+
	5×10^{-5} M	4+	4+	4+
4-hydroxy, 2 keto-butyraldehyde (Synthetic)	5×10^{-3} M	-	-	-
	5×10^{-4} M	+	-	±
	5×10^{-5} M	2+	-	2+
ketoaldehyde from HeLa cells	(calculated)			
	5×10^{-3} M	-	-	-
	5×10^{-4} M	±	-	±
	5×10^{-5} M	2+	±	2+
γ butyrolactone	5.8×10^{-2} M(0.5%)	4+	4+	4+
	5.8×10^{-3} M	4+	4+	4+
	5.8×10^{-4} M	4+	4+	4+

conditioned medium in that the less virulent strains of S. aureus are more inhibited than the more virulent strains. Methyl glyoxal and kethoxal appear to be less active under the same conditions. Since γ butyrolactone is non inhibitory the inhibition by synthetic hydroxyethyl glyoxal is not due to any residual starting material.

(38) Inhibition of Lymphoblast Culture Growth

Purpose:

To test the activity of the purified ethanol extract containing 4-hydroxy, 2-ketobutyraldehyde against the growth of L5178Y cells.

Experimental:

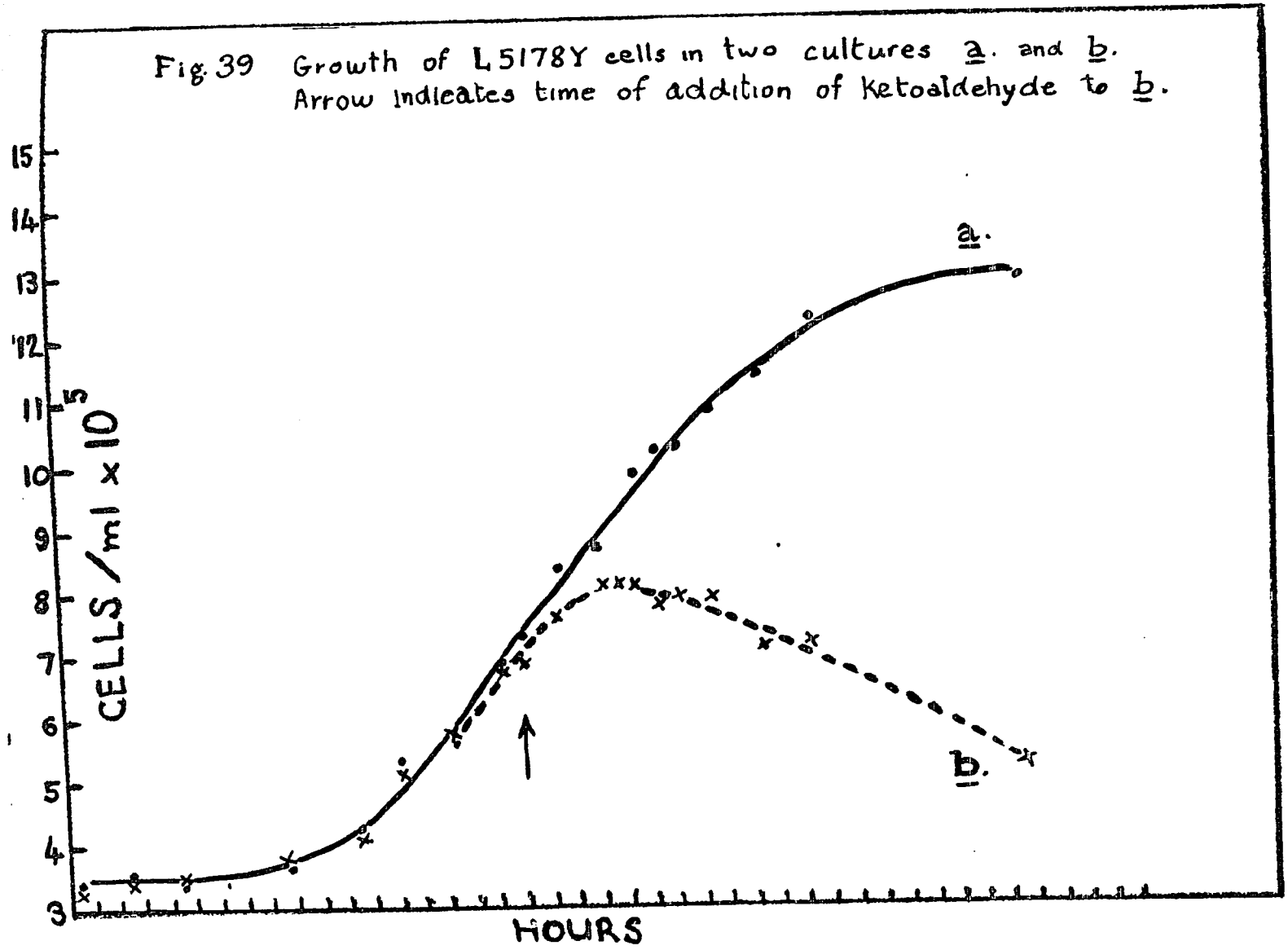
L 5178Y cells were grown in spinner flasks at 37°C with Fischer's medium and 10% calf serum. One culture of one litre was established and when growth was in the logarithmic phase (at the 15th hour) the culture was split into two equal parts a and b, in separate flasks prewarmed to 37°C. Growth of these two cultures was constantly monitored by sampling, counting the number of cells per ml in the total, and in the size thresholds between 10 and 100, and plotting their size distribution. (See methods for settings on the Coulter Counter).

A solution of the methanol-As_2O_3 extract was dried under a stream of N_2 and prepared as in Exp. 31 but the dry residue was taken up in sterile water (2 ml), its O.D. at 276 nm was measured to be 1.433. The solution was added to one of the culture flasks (b) containing 500 ml of culture, at the 18th hour. Two mls of sterile water were added to the other flask (a). The effect on growth was observed by the cell count and size distribution.

Results:

The cell counts of the two cultures are indicated in Fig. 39. The size distribution of the cells is recorded in Figs. 40 and 41. Before addition of the ketoaldehyde the size distributions of cultures a and b were quite similar with a maximum number of cells between size threshold

Fig. 39 Growth of L5178Y cells in two cultures a. and b.
Arrow indicates time of addition of Ketoaldehyde to b.



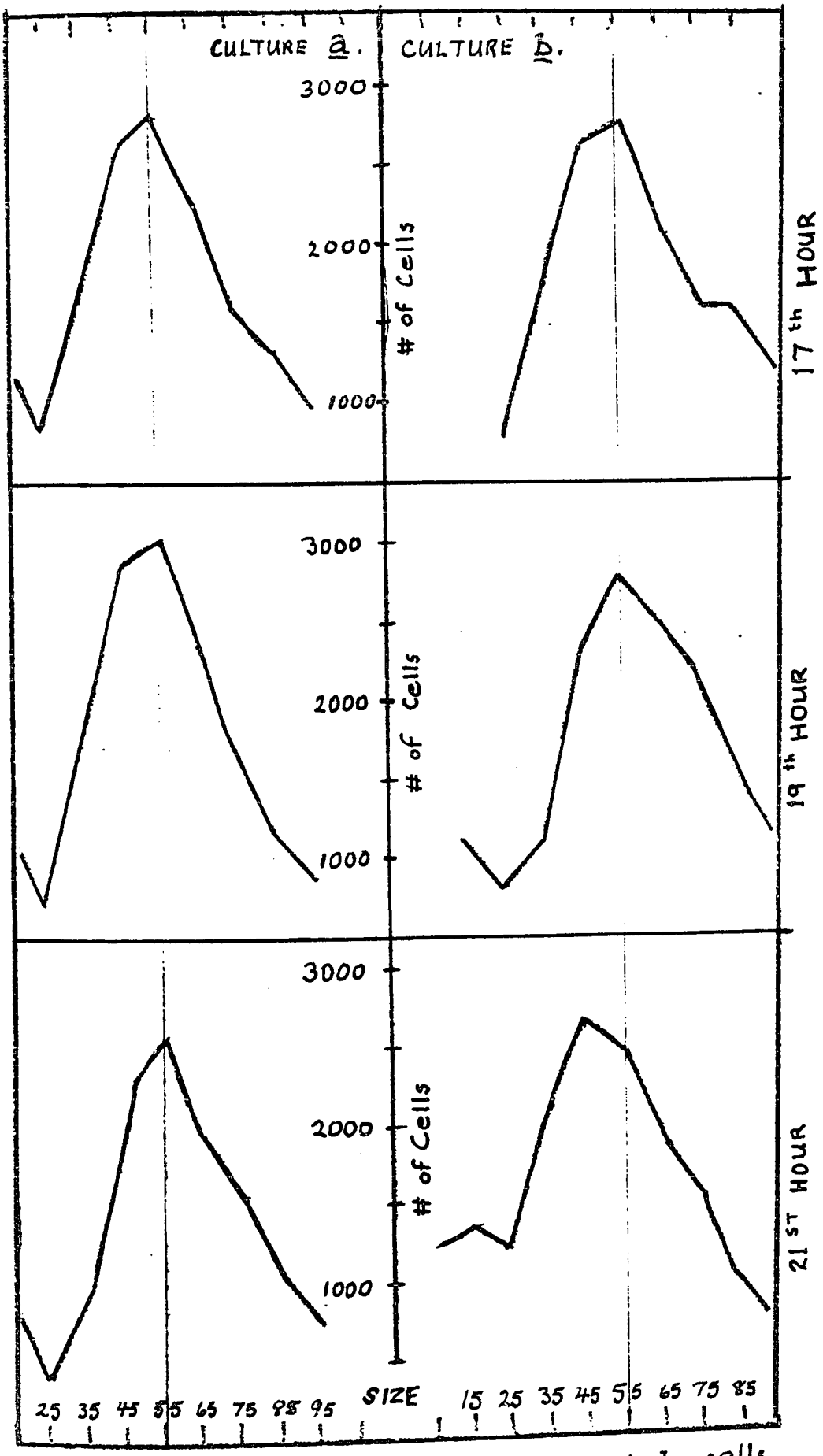


Fig. 40 Size distributions of Culture a and b. cells before and after addition of Ketoaldehyde to b.

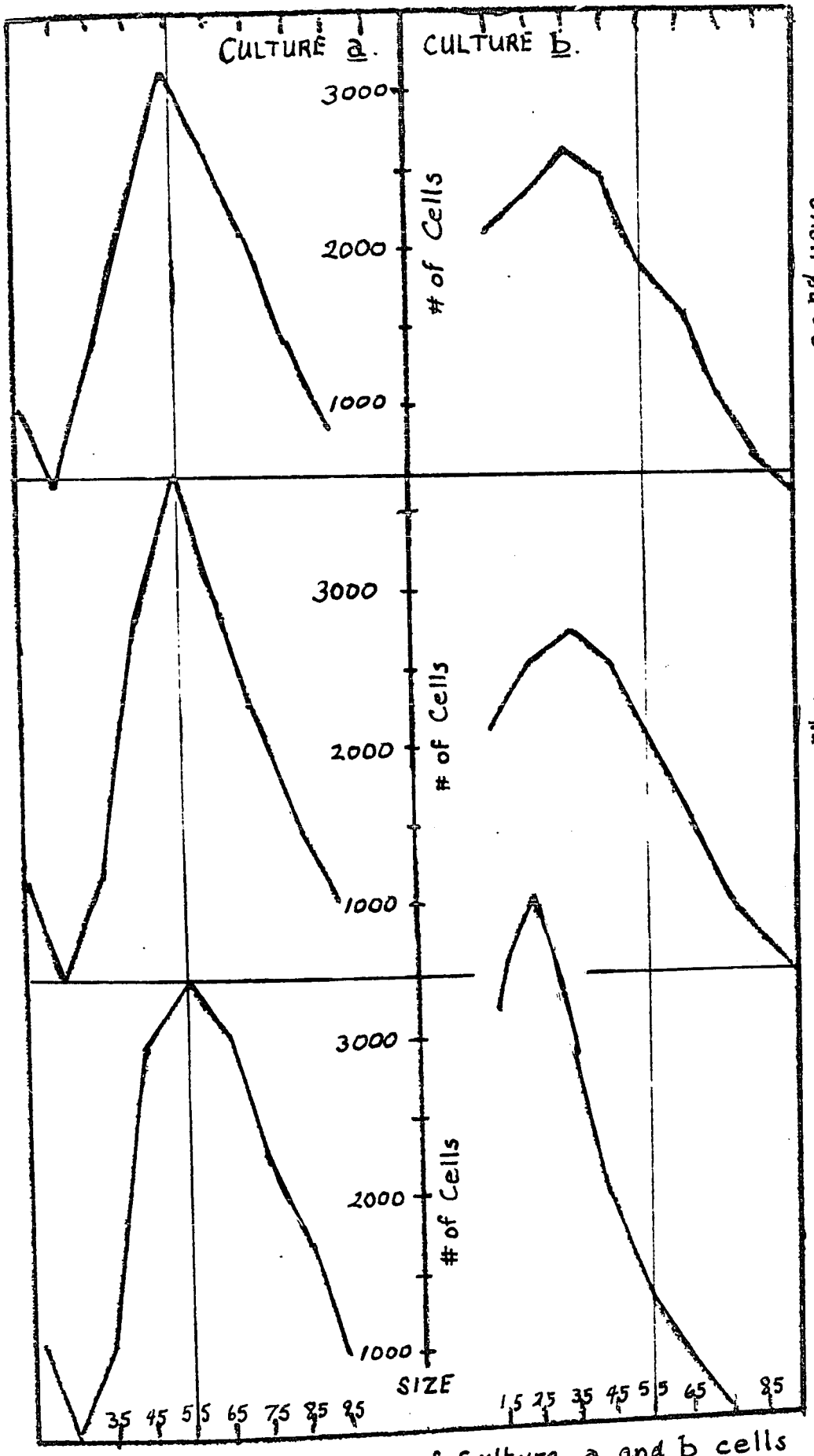


Fig. 41 Size distribution of Culture a and b cells four to seven hours after addition of ketoaldehyde to b.

TABLE 25. Percentage of Culture in Given Threshold Levels

		Hour of Growth						
CULTURE A.								
Threshold	Cubic Microns	14th	17th	19th	21st	22nd	23rd	25th
10 - 20	275	9.3	8.3	7.7	7.8	7.4	6.8	6.6
20 - 30	458	3.9	3.5	3.4	3.3	3.4	3.0	3.3
30 - 40	641	10.6	10.6	10.9	8.1	8.9	6.8	6.5
40 - 50	825	16.8	18.7	19.7	18.0	17.7	16.4	17.4
50 - 60	1006	19.1	20.1	20.6	20.3	19.2	22.0	19.8
60 - 70	1190	14.7	16.0	15.6	15.4	16.2	17.3	17.3
70 - 80	1375	11.4	10.5	10.8	12.5	15.0	13.0	13.0
80 - 90	1557	8.9	7.7	6.9	8.6	8.6	8.6	10.3
90 -100	1740	6.0	4.6	4.4	6.0	5.6	5.7	5.9
CULTURE B.								
Threshold	Cubic Microns							
10 - 20	275	7.9	6.9	6.5	9.5	14.0	13.8	20.0
20 - 30	458	3.2	3.1	3.8	9.0	15.6	16.7	23.8
30 - 40	641	9.5	11.1	6.5	15.2	17.2	18.4	18.0
40 - 50	825	18.6	18.8	16.0	18.9	16.3	16.9	12.1
50 - 60	1006	19.8	20.0	19.9	17.7	12.5	13.5	7.9
60 - 70	1190	16.2	14.3	17.9	13.9	10.3	9.3	5.4
70 - 80	1375	12.1	9.8	15.2	11.2	7.1	4.9	3.7
80 - 90	1557	8.2	9.6	10.4	7.1	4.5	4.3	1.7
90 -100	1740	4.4	6.4	7.0	4.8	2.6	2.6	1.0

settings of 50 and 60, and nearly that same number between 40 and 50 as seen in Fig. 40 a and b for the 17th hour. These are ideal cultures and ideal instrument settings. One hour after the addition of the ketoaldehyde to b the cells seemed to swell significantly as seen by a rise in the number of cells within the size thresholds 60-70 and 70-80 (b at the 19th hour, Fig. 40). By three hours after addition, the total number of cells within sizes 70-80, 60-70, and 50-60 had dropped. The peak size was therefore between 40 and 50 (b at the 21st hour, Fig. 40). By the fourth and fifth hours after addition of the ketoaldehyde the number of smaller 'particles' rose significantly at the expense of the larger ones, (b at the 22nd and 23rd hours, Fig. 41). As the total number of cells per ml showed a decline (Fig. 39) this loss of the large cells can be explained by their bursting and breaking into smaller particles while smaller cells could remain unaffected. Many of the smaller particles were below the lower threshold setting of 10 and were not counted.

Conclusion:

The purified inhibitor stops the growth of the L5178Y cells measurable by the second hour after its administration. The size of the cells becomes much larger in the first hour, as if by swelling, then smaller as if large cells were breaking up into small pieces of debris. This type of action could resemble an agent acting on the membrane.

D I S C U S S I O N

1. THE PHENOMENON OF GROWTH INHIBITION

A. Bacterial Growth

The work reported in this thesis grew out of an observation by Dr. C.P. Kenny, made during studies on the host-parasite relationships of human tissue culture cells and bacteria. A variety of strains of E.coli were inoculated into HeLa cell monolayer cultures in antibiotic free medium to provide a system for studying the infection process. Normally the bacteria grow exceedingly well in fresh tissue culture medium but in most of these experiments bacteria failed to grow. Those unsuccessful strains would not survive in the tissue-cell used medium after it had been separated from the monolayer that had been nourished by it. This finding indicated that the growth inhibition did not depend on the physical presence of the human cells. The cell-conditioned medium (CCM) therefore either lacked some nutrient essential for growth of the bacteria or contained an inhibitor of bacterial growth. In preliminary experiments CCM was supplemented with fresh medium to provide adequate nutrition, yet the bacteria still failed to grow; this was taken to indicate that the latter alternative was true: an inhibitor was present.

Ascertaining the chemical nature of the inhibitor required first its purification. Since the inhibitor was known solely by its antibacterial activity a bioassay was the only method of tracing this activity during purification. To take into account any variation in the strength of the inhibitory activity of CCM the bioassay was designed to 'titrate' the activity. Testing a series of tubes of CCM (1 ml volume each) by inoculating decreasing numbers of bacteria (E. coli serotype O14,Table 1) the strength of the inhibitor was monitored. Usually the 1 ml of used medium inhibited the growth of E. coli O14 if their numbers were less

than 10^6 in the 1 ml. Larger numbers grew more slowly than the controls but nevertheless survived.

i. E. coli

To ascertain that inhibition was a more general phenomenon a variety of E. coli strains was assayed for growth in the presence of CCM. Some of these strains had been collected from cases of diarrheal disease caused by E. coli. Such strains are therefore by definition, virulent pathogens and the only method of distinguishing them from non virulent E. coli had been serotyping, since biochemical tests gave always identical reactions (103). The Communicable Diseases Center in Atlanta Georgia had established a list of E. coli serotypes associated with diarrheal diseases and the designation Enteropathogenic E. coli was suggested for these particular serotypes by the American Public Health Association Committee on Enteropathogenic E. coli. A distinction between the virulent and non virulent serotypes was seen in Exp. 2 as their differential ability to grow in CCM. Those serotype strains that were inhibited less had a known disease producing capability (serotypes 026, 0126, 0127, 055, 0119). But even these latter were inhibited to some degree in that they grew slower than controls.

The inhibition was more effective against the less virulent strains. The implication of this phenomenon was that the degree of virulence of an organism pathogenic for man may depend on its resistance to an inhibitor produced by human cells. To see if this was the case amongst other genera a variety of bacteria, pathogenic for man, was selected, whose degree of virulence was already established by standard criteria.

ii. S. aureus

In the first case Staphylococcus aureus has been classified

according to phage type groups I, II, III, IV, and Miscellaneous. These classifications were established by Blair and Williams (42). By phage typing strains isolated from wound infections, pneumonia, and bacteremia, and comparing them with those carried in the nares of healthy outpatients, McCabe found (213) (214) that phage groups I and III were associated with the disease cases. Independent confirmation of this association of I and III with clinical staphylococcal infections, and with an apparent increased resistance to chemo-therapy, has been made by Duncan and Comptois (88). It was accepted that overall virulence of strains producing human infections is probably greater than that of strains isolated from healthy carriers. However it was recognized by McCabe (214) that generalizations concerning virulence in human infections are not necessarily valid without considering host factors in the development and outcome of staphylococcal infections. 'Host Factors' may refer to such substances as the inhibitor found in human cell cultures in these studies.

Many other parameters had been studied in relation to the disease producing ability of staphylococci but no definite correlation to disease production other than bacteriophage type, had been established between antibiotic sensitivity, (31) quantitative coagulase production, production of pigment, gelatinase, or lipase. It had not been possible to define a single factor responsible for the virulence of staphylococci although several had been excluded. Some investigators had suggested that clinical virulence correlates more closely with the total number of factors rather than any single factor (73).

Be that as it may, it only shows that many of the determinants of pathogenesis remain imprecisely defined despite their increasing importance.

McCabe therefore established (213) a laboratory test sensitive enough to compare the relative virulence of strains of staphylococci. By infecting embryonated eggs, a distinct relationship between pathogenicity of staphylococci for humans and their virulence for chick embryos was observed. Strains of S. aureus definitely responsible for human infections were extremely lethal to chick embryos. Other strains of indeterminate potential pathogenicity to man isolated from uninfected carriers exhibited individually variable degrees of virulence in the chick embryo but as a group were significantly less lethal than strains isolated from clinical infections. Infection of strains of S. epidermis, non-pathogenic for humans were no more fatal to embryos than control injections of culture media and therefore were considered non lethal for embryos. In embryos again phage groups I and III were established as more virulent than groups II and IV and Miscellaneous.

iii. Neisseria Strains

In exp. 3 Table 3 it is observed that strains of S. aureus from groups I and III survived the inhibition suffered by groups II and IV and M strains. Because growth of virulent and non virulent E. coli serotypes is also distinguished here, the data demonstrated, for the first time, that growth in the presence of the inhibitor, characterizes the degree of virulence in more than one genus. This was borne out again in Neisseria gonorrhoeae where colony type 1, the virulent form, survived the inhibition undergone by avirulent type 3. The establishment, in N. gonorrhoeae, of colony typing associated with virulence had been done by Kellog et al.(161) (162). Again the criterion of virulence was the production of disease in the human. In primary isolates from acute gonorrhoea in males 90% were type 1, 10% were types 2 and 3. Kellog described the visible

characteristics of all colony types and pointed out that the 4 types have been found to represent the most permanent and most significant morphological character under specified conditions of cultivation . After unselective laboratory passages of organisms from primary isolates, predominantly type 1, on appropriate medium, types 3 and 4 became more dominant. Type 3 then became a minority of the population composed predominantly of type 4. Type 1 was therefore lost on passaging. Type 2 could make a rare occasional appearance for some months after preliminary isolation.

If type 1 colonies were specifically selected for subculture any one or more of the other 3 types appeared along with 1. In an early study (160) this happened in 80 selective transfers of type 1. It suggests a genetic instability of type 1 morphology in vitro. Yet CO_2 and iron tended to promote the stability of types 1 and 2 under in vitro conditions. After 720 selective transfers colonial morphology of types 1 and 2 still remained closely related to virulence.

The degrees of virulence of the types were determined by their ability to cause disease in man. Experiments were established using human volunteers (161). Inoculating them with type 1 always produced an infection if the type 1 was selected continually. Type 2 would also produce the disease but not type 3. Type 4 laboratory strains did not produce an infection but type 4 could produce the disease if selected directly in one subculture from a type 1. However this virulence of type 4, selected from 1, did not appear after 38 passages of 1. It was concluded that virulence was associated with the type 1 heritable clone, was maintained in type 2 but was lost in 3 and 4. These studies are some of the few defining virulence of an organism by the deliberate

infection of man. Virulence as thus defined was found to be associated with the ability of colony type 1 of Neisseria gonorrhoeae to survive the inhibition by the bacterial growth inhibitor from human cells(table 3). However all the Neisseria meningitidis strains tested this way were inhibited, but they were laboratory strains and their virulence was unknown at the time. Later when fresh isolates of N. meningitidis were available and a test was established for experimental infections in mice, data concerning virulence became available. By this time the hypothesis, that the degree of virulence of a pathogenic organism depends on its resistance to the inhibitor, could be tested further by correlating the survival of an organism in CCM to its mouse killing ability.

Laboratory strains of N. meningitidis of serotype A, B, C, or D all gave an LD_{50} in C 57 mice of 10^7 organisms, of types X,Y, or Z the LD_{50} was 10^6 organisms (exp. 4). Strains of these serotypes isolated from disease cases gave LD_{50}'s of 10^1(553 Y and 247 X), 10^2 (13 C and 552 B), 10^3 (604 A) and 10^4 (564 Z), all indicating a higher virulence than the lab. strains (Exp. 4). This distinction was borne out in the CCM survival assay (Exp. 5) where laboratory strains were inhibited in greater numbers than disease-isolated strains. To show the correlation between LD_{50} number and CCM Survival Number (Exp. 5) the data from tables 4, 5 and 6 were collected into table 25. A graph, Fig. 42, depicts these values around a straight line. Near the origin lie virulent strains, away from the origin lie the less virulent strains. Strain 129 A, although isolated years ago had been lyophilized since isolation and samples were available after relatively few passages on lab. medium. Its intermediate virulence is seen on the graph (Fig. 42) half way between virulent and less virulent organisms. Z serotype strains have

been rarely implicated in disease cases and only isolated from healthy carriers. A few exceptions however were recently reported, (5). Their general lack of virulence can explain why 564 Z is half way between virulent and less virulent organisms on the scale depicted in Fig. 42. The correlation of the measure of virulence in mice with the resistance to inhibition by the HeLa cell factor seems to support the view that the inhibitor is definitely capable of discriminating virulent strains of a variety of bacterial genera from less virulent strains. The factor therefore could provide a tool to examine the nature of bacterial virulence.

Since the publication of the virulence test with HeLa cell used medium reported in this thesis (166) others have confirmed the finding that virulent bacteria can be distinguished from avirulent bacteria in HeLa culture. Calabi (74) demonstrated that virulent shigellae invaded and killed the monolayer cells while avirulent strains did not invade or multiply. Also non-pathogenic E. coli strains were killed in the HeLa cultures. Even more recently the studies of DuPont et al. (89) with E. coli confirmed the ability of HeLa cell cultures to inhibit the avirulent strains but not the virulent strains. DuPont et al. found that E. coli strains causing guinea pig enteritis and a positive kerato-conjunctivitis (Sereny test) also invaded HeLa cells, while strains incapable of causing these diseases did not invade HeLa cells and had no apparent effect on the culture.

In light of these confirmatory studies the phenomenon of growth inhibition by HeLa cell culture conditioned medium and the differentiation of virulent and avirulent bacteria suggests to us therefore, a general principle about the nature of virulence.

TABLE 26 The number of organisms of N. meningitidis giving an LD_{50} and surviving in CCM, taken from tables 4, 5 and 6.

STRAIN	LD_{50}	CUT OFF POINT (Lowest # in inoculum, which survives inhibition)
1027A	10^7	10^6
2092 B	10^7	10^5
1628C	10^7	10^5
158D	10^7	10^5
SLAT X	10^6	10^4
SLAT Y	10^6	10^4
SLAT Z	10^6	10^4
129A	10^4	10^4
604A	10^3	10^3
552B	10^2	10^2
13C	10^2	10^3
247 X	10^1	10^2
553 Y	10^1	10^2
564 Z	10^4	10^3

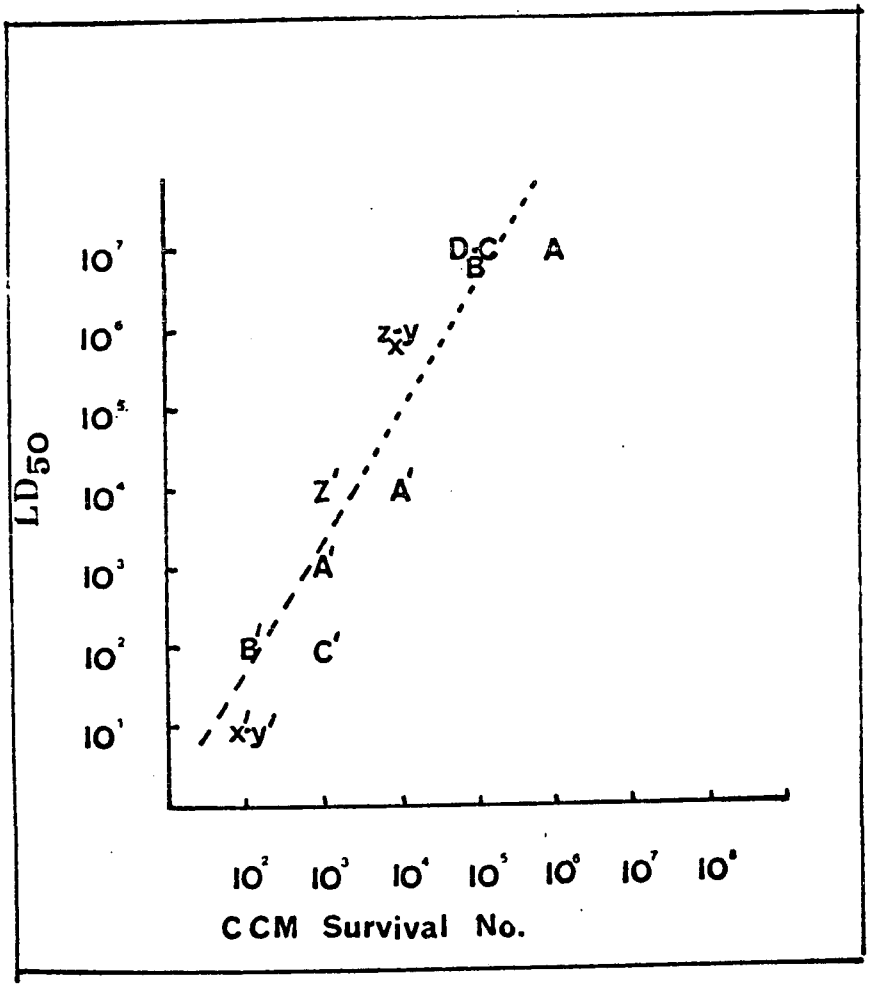


Fig. 42

Strains of N. meningitidis by serotype as listed in Table 26, plotted by LD_{50} and CCM survival number. The 'prime' indicates a virulent strain.

B. Virulence

i. Bacterial Characteristics

The near synonymous terms pathogenic and virulent are used in this work in a specific way; the former refers to species and the latter to degrees of pathogenicity of strains within species. Virulence itself has appeared so far to be related to the resistance of the organism to the inhibitor from human cells. Experiment 7 showed that although both virulent and less virulent strains were capable of lowering the amount of inhibitor in CCM, perhaps by destroying it or binding it, the virulent strains were not any more capable of cutting down inhibitory activity than were the less virulent strains; the opposite appeared to be the case. Therefore virulence was taken to be the ability to avoid inhibition by an environmental factor rather than ability to destroy or bind the inhibitor. This is a new point of view.

In the past, virulence has usually been defined as the ability of microorganisms to multiply in a host and to cause a progressive disease, while avirulent strains not only fail to multiply but are rapidly eliminated from the tissue by the host. As well, an organism may be virulent for one host species and avirulent for another (43) (285). Such definitions point out the importance of the host response in the course of any infection, and therefore imply that virulence involves the organism's reaction to the host's response.

Host resistance must be a crucial factor in determining virulence. Infectious diseases caused by microorganisms belonging to the normal flora of a host and generally regarded as non-pathogenic, have been designated opportunistic infections (171) or as reluctant pathogens (163). These terms imply some derangement in normal host defence mechanisms. In fact

deficiencies in host resistance have been thought to confer the potential for pathogenicity on to every microorganism (171). Yet it is more often believed that the organisms themselves have the capacity for virulence or do not have it. In particular the compounds in the cell wall complex: those responsible for resistance to cellular defenses, for resistance to humoral defenses, and those that are toxic to host tissues, are thought to confer virulence. Capsular polysaccharides such as type specific pneumococcal (20) ones, the hyaluronic acid capsule of streptococci (29), and the poly-D-glutamic acid capsule of B. anthracis are important virulence factors (317) by dint of their antiphagocytic properties (108). The M protein of streptococci also protects against phagocytosis (29) as does the O antigen polysaccharide of E. coli (216). But how would these wall materials protect the virulent bacteria against other host defences, for example, the inhibitor from human cells?

If the virulent organism seems relatively inert to the inhibitor from human cells it may have some protection around it, and the cell wall or capsule comes to mind. Rowley (263) has explained quite well how the amount of the oligosaccharide on the surfaces of strains of Salmonella and other genera can determine whether the organism is poorly or easily destroyed by either antibodies or phagocytes; the more capsular material the less exposed is the vulnerable cell membrane and rough-core antigens. Thus 'rough' (R) strains are avirulent, and 'smooth' (S) strains, glistening colonies of encapsulated cells, are classically virulent. The capsule could prevent a growth inhibitor from entering the cell membrane just as well as it protects the membrane against antibodies or phagocytes. The designation of these varients as R forms and S forms is common. The virulent S form, upon subculture in artificial media becomes progressively less smooth and will later appear rough and unencapsulated. The change

S → R is often a reversible one provided the R cells are not too far 'degraded' (21). The reversion of the R form to its original S type can frequently occur by successive animal passages or by repeated subculture in medium containing antiserum to R. When reversion occurs under these conditions the R cells always revert to the S form of the same specific serotype as the R. The reversible conversion of S ⇌ R of one serotype is quite different from the genetic transformation of one serotype into another. The former cell forms are referred to as variants and arise gradually while displaying degrees of encapsulation and of virulence; the latter are mutants or transformants and appear in one passage, or spontaneously.

That variants should arise on artificial media and revert in vivo obviously indicates the effect of the environment on virulence and morphology. The gradual loss of virulence during laboratory culture of virulent organisms recently taken from a living animal has been observed with Neisseria strains in these studies and with many other bacteria (86). In particular the difference in virulence between smooth and rough strains of Vibrio cholera appeared to be consistently and quantitatively due to the presence of the O antigen, O being essential to virulence and smoothness (185). Hemolytic streptococci have also been studied in this regard; the glossy colonies of the avirulent strain have lost the M protein while the matt colonies of the virulent strains contain the M protein. Although the streptococcus has a hyaluronic acid capsule as well, which was also quantitatively related to the degree of virulence, the M protein was deemed more important as a virulence factor (187).

The virulence of pneumococci depends on a specific capsular polysaccharide on the surface of the cells (21) (128). The chemical nature

of capsular material is genetically controlled in virulent strains but the capsule itself, when isolated, has proven to be non toxic and therefore its involvement in virulence is not as a destructive invasive instrument, but perhaps as Rowley (263) suggests, is a protection for the bacteria against host defenses. Although the chemistry is genetically controlled, the quantitative expression of a capsule has been shown to be altered by environmental conditions. Dietary Mn^{++} in the host was found to be a specific naturally occuring environmental factor that enhanced virulence of pneumococci by encouraging encapsulation (145). That the potential virulence of pneumococci can be controlled by the physico-chemical characteristics of the environment has been suggested by a number of investigators for many years (87) (339) (39).

The virulence of an organism has experimentally been increased by animal passage of initially avirulent organisms. Virulent variants eventually arise in the in vivo environment. This fact implies that environmental substances and processes connected with virulence occur in vivo to encourage or stimulate the growth of the virulent type. Although the turning of avirulent pneumococci into virulent organisms of a different serotype in vivo (128) was a genetic transformation (21) the change of an avirulent type to a virulent strain of the same type by DNA from virulent cells was under careful investigation (57) (110) (111). Enzymatic digests of DNA derived from either bacterial or mammalian sources were found to stimulate bacterial changes from avirulent towards virulent cells in vitro. DNA treated with DNase promoted changes from R to S in Diplococcus pneumoniae and in Brucellus abortus. In the former, natural breakdown products such as deoxynucleotides and deoxynucleosides had to be added for optimal effects on the R to S change. However a factor

in the digests not identical with known breakdown products, was still responsible for the change (56). In a search for other factors to enhance the R to S change it was found that Mn^{++} but not other divalent cations was significant. In the absence of this ion no selective effects were observed despite the presence of the digest (112). What then, are the environmental influences which determine whether a microbe is virulent or not?

ii. Environmental Effects

In Bacillus anthracis two factors certainly associated with virulence were the capsule and the toxin production. The capsule (316) consisted of a glutamyl polypeptide and its synthesis depended on the ion composition of the medium, Mn^{++}, Co^{++}, and Zn^{++} having an effect as in B. subtilis capsule synthesis (188). Thus encapsulated virulent strains would yield unencapsulated avirulent strains in medium lacking ions and poorly encapsulated cells in medium of limiting ion concentration. The virulence of B. anthracis had been recognized as not only due to the presence of the capsule but to another factor not associated with capsule (298). In addition CO_2 could encourage the capsule to grow in vitro and its absence permitted the appearance of rough colonies. Such environmental effects as those of ions, CO_2 and pH on the capsule, and presumably on virulence, emphasize the differences between in vivo and in vitro growth (56), and imply a physiological, non-hereditable adaptation to environmental conditions rather than genetic mutations.

Diptheria bacilli also give rise to smooth and virulent, and rough and avirulent colonies, but smoothness does not correlate with toxin production. It does however correlate with iron concentration in the environment (346). Iron has been implicated in the virulence of

N. gonorrhoeae (160) in the stabilization of the freshly isolated Type 1 colony in passaging in vitro, and iron has also been capable of turning colony type 3 or 4 (avirulent) into type 1 (virulent) by incubation in vitro (163). Type 1 colonial morphology in the gonococcus is said to be genetically unstable (160). The virulence of the diptheria bacillus is said to be under genetic control while the gene governing toxin production resides in certain phages (27) (28). Can iron therefore effect the genetic expression of virulence?

Often a multivalent cation has been implicated as an environmental agent significant in the genetic expression of what were believed to be properties of virulence. Iron has been studied in the resistance to infection by Clostridium welchii (261), and was found to have effects on bacterial growth depending on the Eh of the tissue, which state determined the oxidation level of the iron. Iron enhanced the virulence of Pasturella pestis (151) of Pasturella septica and of E. coli in guinea pigs (60).

The fact that certain characteristics of virulence (eg: colonial morphology type 1 in N. gonorrhoeae), are unstable and are lost on repeated passaging in vitro could mean that the phenotypic expression of virulence was adaptively repressed. This would be possible since the reappearance of the virulence characteristics in an avirulent organism selected in one passage after incubation with iron (eg: gonococcus) or in other examples with manganese or zinc, or passaging in living animals, would suggest that the genetic control of virulence was never absolutely lost from the organism but was repressed due to environmental influences. However some strains seem to have permanently lost their S character (eg: pneumococcus R36 A (21) and R 36 NC (20)), and are used

experimentally to advantage.

It seems clear then that the effect of the environment and thus of the host defenses on determining virulence of an invading organism may be fundamental to the nature of virulence.

iii. Research on Virulence

There has been great difficulty in interpreting and investigating biochemical mechanisms of pathogenicity. The main factor was that virulence had been measurable or detected only as the success of an organism in vivo and this had been markedly influenced by changes in growth conditions of the same organism in vitro where variants with phenotypic changes may be selected (219). There are often avirulent strains of a pathogenic species which are indistinguishable from the virulent strains in most available tests in vitro so virulence is determined only by small differences expressed during growth in vivo. Bacterial behaviour in vivo is nevertheless not easily examined. The occurence of the inhibitor described in this work is the first indication that virulence of strains, in all species tested, is measurable as survival of the organism in its presence. The inhibitor presumably could be responsible for host or tissue resistance in the host or tissue that made it, and indeed differences in susceptibility to infection occurring between different hosts or tissues are well known. It has been pointed out that "if the chemical basis of resistance of a certain species (or tissue) to infection by bacteria could be identified it might be possible to confer on an otherwise susceptible species a higher level of resistance than that produced by immunization... Undoubtedly variations of bactericidal mechanisms in different species and tissues could account for differences in specificities of infection",

(293).

The existence of common features between the behaviour of malignant cells and the pathogenic microorganisms has been noted (114) (270). Malignancy, the ability of cancer cells to invade and kill a host, like microbial virulence, appears to vary and to depend on genetic and environmental factors. Thus cancer 'strains' of differing malignancy exist and malignancy can be increased by animal passage, and decreased by culture in vitro (114) (270) (294). Just as the chemical composition of bacteria is subject to considerable phenotypic modification depending on the environment as Collins found (76) with fermentor cultures, so mammalian tumor cells similarly change depending on their in vitro culture conditions (295).

Additional similarities between cancer and infectious diseases include the importance of nutrition for the host, for example the effect of serum aspariginase on leukemias, (52) and host defence mechanisms in tumor growth, the tendency of cancer cells to grow better in some tissues than others, and the production of active immunity by malignant cells. There is even a possibility that cancer cells produce toxic factors (301). The host resistance then is an important factor, and the chemical basis of resistance is therefore valuable to know. Identification of the inhibitor from HeLa cells was therefore pursued as the most important goal of this work.

C. The Inhibitor

i. Occurrence

Experiment 7 demonstrated that the differential growth response of virulent and less virulent S. aureus to the inhibitor can be demonstrated on agar plates through diffusion of the inhibitor from paper disks. Since strains of S. aureus are definable as virulent or less virulent according to phage type and since virulence in these organisms has not been found to be lost through passaging on lab medium, S. aureus was chosen as the test organism for further assays of virulence and of the inhibitor.

In testing the production of the inhibitor by the HeLa cells it was observed that the quantity increased daily up to 3 days at least (Exp.8). The cells could produce it in Eagles Minimal Essential Medium, or Medium 199, or Heart Infusion Broth with yeast RNA. Seven different human cell lines, free from PPLO infection, were able to produce an inhibitor of the same discriminatory nature but two monkey cell lines did not, nor did two human lines infected with PPLO. It is not possible to explain why monkey cells cannot inhibit the bacteria which are human pathogens but since this data is so little it is best not to speculate at all. At least all the human lines used behaved the same way in so far as they produced some inhibitor or inhibitors, unless infected with PPLO, (Exps. 10, 21). In this regard it is interesting that the infection prevented inhibitor production. PPLO, or any infectious agent can get into the cells early in the age of the monolayer cultures, when the medium is fresh and before the inhibitor has accumulated in it.

ii. Isolation

Before isolating the inhibitor its relative stability was investigated to learn whether it would be necessary to avoid certain

treatments in the purification process. The inhibitory activity turned out to be stable to drastic pH changes, and to extremes of temperature. The fact that activity survived autoclaving for a short period of time suggested that the inhibitor might have a low molecular weight; indeed it was found to be dialysable. This gave an indication of the size of the factor and Sephadex G 50 was used first to chromatograph concentrated CCM. G 50 excluded all material of molecular weight over 50,000 thus getting rid of the calf serum proteins of the medium. The choice of monitoring the column effluent at a wavelength of 280 nm is arbitrary. As it is unknown a priori what the nature of the inhibitor is, there is no reason to choose a particular wavelength nor to assume even that the inhibitor absorbs light. Nevertheless monitoring at 280 nm does allow for the fractionation of bulk material into several parts and thus provides at least some method of separating inactive material from active material. The third peak of the G 50 column was active in inhibiting the test organism (Exp. 14) and since no inhibition was seen with peak II peak III was taken as containing most if not all of the inhibitor.

Since these peaks comprised concentrated material, and peak III in particular contained the phenol red from the medium, a control of fresh medium was fractionated. Here the third peak with phenol red, concentrated to the same degree, had no inhibitory effect. After passing the active fraction through a column of Sephadex G 25 the activity was eluted after the void volume in the first of two peaks and was once more separated from inactive material appearing in the second peak which, incidentally, contained the phenol red. After passing the active G 50 fraction over Sephadex G 10 the void volume peak displayed the activity indicating that the molecular weight exceeded about 700. When active material from

the G 50 fractionation was passed through Sephadex G 15 the inhibitor was eluted immediately after the void volume indicating a molecular weight below 1500.

iii. Nature

At this stage speculation on the nature of this material remained as open as it had been at the beginning, and to identify the inhibitor from used HeLa medium it was necessary to consider carefully its known properties for a clue to its unknown chemical nature. First of all the mere fact that it is secreted into the medium and not retained by the human cells suggests that it can exert an action against the growth of organisms or neighbours in a wide area around the cell that produces it. Although it is found in all the human cell lines tested one could argue that it is a phenomenon of established tissue culture cell lines which have adapted to a particular way of life. But the failure of other tissue culture cell lines (monkey cells) to exhibit the same phenomenon, and the presence of the inhibitor in primary human amnion cell cultures, five days out of the adult human, suggest it is more than that, and the specific behaviour of preferentially inhibiting the less virulent strains of a variety of bacterial genera indicated it could be an important normal physiological substance.

The type of inhibition observed with bacteria is not an all or none phenomenon. Although the more virulent strains survive the inhibition they still grow more slowly in the presence of the inhibitor than control cultures, so they are inhibited to some degree. It is not as if the inhibition were instantly pernicious. The ratio of inhibitor concentration to number of bacteria is important for the evaluation of growth inhibition, the fewer the numbers of virulent organisms the slower the

growth while very large numbers of less virulent organisms survived and grew in the presence of the inhibitor. This would clearly indicate that the bacteria have a means of overcoming or avoiding the inhibition, some more than others as seen in experiment 6.

The question begging an answer was whether there were any precedent for a compound with quite these properties.

It seemed reasonable that human cells should have some protection of their own against growth of organisms in their environment; tissue and host resistance besides immunity is well known. This principle of resistance has been exemplified in the action of antibiotics from moulds but most examples of these growth inhibitory substances relate generally to killing all strains of one type of organism and not specifically virulent strains more than others of the same species.

A growth inhibitory substance from clams and mushrooms was reported, and was speculated to deter the growth of parasites on these organisms, (304). It was found in a variety of animal tissues including human urine. Because it retarded growth of tumors and tissue culture cells it was named retine. Early in the investigations on retine the assumption that retine was one substance and not many was made as a judgement of its single behavioural characteristic: growth inhibition. As it was later identified as having a ketoaldehyde for its active group other keto-aldehydes were examined in comparison. Fertilized sea urchin eggs, seeds, flagellates, tissue culture cells and bacteria were all inhibited by methyl glyoxal (96). Methyl glyoxal, the simplest ketoaldehyde, was shown to inhibit the growth of E. coli reversibly and to inhibit in amounts quantitatively relative to the number of E. coli. The inhibitory action of the aldoketone depended less on its concentration

than on the relation of its quantity to the number of cells acted upon. This suggested it reacted in stoichiometric fashion with some grouping in the cell. The cells seemed to compete for the inhibitor thus protecting one another (96) so that large numbers had a better chance of survival.

Such an activity was suspiciously similar to that of our factor. Further than activity alone there was a similarity in physical properties. Retine was carried on a carrier, presumably a polypeptide, which, by Sephadex fractionation behaved as if it had a weight of 1000 (136). A small ketoaldehyde like methyl glyoxal for instance, could be stable, especially attached to a carrier. The likeness of retine to the inhibitor was sufficient to warrant testing the factor for ketoaldehyde-like characteristics. The simplest indication was to study whether cysteine would reverse the inhibitory activity of the factor as it can reverse the inhibition by methylglyoxal (94), and also to test the effect of methyl glyoxal to see if it would mimic the inhibitory action of the factor. Both tests confirmed the suspicion that bred them(Exps. 18 and 19). Cysteine reversed the inhibition by the factor and methylglyoxal inhibited S. aureus strains from groups II and IV more than those of groups I and III. But as this latter distinction was made only at the first five hours and not at 15 it appeared that methyl glyoxal is less effective than the unknown factor in inhibiting growth.

With suggestive evidence that the unknown inhibitor might be a ketoaldehyde it was feasible to attempt to isolate one directly from the active Sephadex G 25 fraction. As suggested by Szent-Györgyi in extracting ketoaldehydes from tissues the use of As_2O_3 and methanol frees the dicarbonyl from a carrier presumably attached through -SH

groups (304). The use of As_2O_3 in expt. 20 did liberate the antibacterial activity from a methanol insoluble material to a methanol soluble one and the active supernatant was yellow-brown the colour of aldoketone solutions. This result implied that the activity was due to something which competed with As_2O_3 for the same certain group, and strongly supports the assumption that a ketoaldehyde is attached to a certain carrier by an -SH group. As Szent-Györgyi warned (113) (305) As_2O_3 can free glucosulose from a glucose-amino acid present in crude tissue preparations and the reactions of ketone and aldehyde would be positive, interfering with the identification of a ketoaldehyde growth inhibitor. However in experiment (20) the activity only was assayed not the chemical grouping aldoketone. The active Sephadex G 25 fraction used in experiments 20 and 21 should in any case be lacking glucose-amino acids as they would be chromatographed with lower weight material. The proof that no glucose-amino acid or other unnatural precursor of aldoketone is present in such a fraction, and that no artifact came from the treatment of this fraction with As_2O_3, was found by treating a similar G 50 & G 25 fraction from similar medium conditioned by PPLO-containing HeLa cells (Exp. 21), with As_2O_3 and methanol. Here no antibacterial activity was present yet the medium had supported cell growth. Where activity was lacking in this conditioned medium there was no activity in the CH_3OH-As_2O_3 extract either, and the solutions were clear. The yellow colour of the active extracts seemed to correlate with antibacterial activity and its absence correlated with no antibacterial activity. The colour was reminiscent of solutions of methylglyoxal and U.V. absorption (Fig. 7) indicated this. The absence of U. V. absorption (Fig. 8) in the clear, non active extract from uninhibitory medium conditioned by PPLO

infected cultures confirmed that As_2O_3 was definitely not causing the production of an artifact in exp. 20.

Up to this point it has been suggested that the inhibitor from HeLa cell conditioned medium behaved as a ketoaldehyde. The overall purpose of this thesis was to identify the inhibitor chemically, therefore, to prove that it was a ketoaldehyde or not, an understanding of ketoaldehyde chemistry, and of the means of identifying such compounds by the interpretation of spectra was necessary. The following description of the carbonyl group provides a basis for understanding the spectral absorptions which are discussed afterwards.

2. CHEMISTRY OF DICARBONYLS

A. Carbonyl Structure

The simplest carbonyl compound is formaldehyde. In the formaldehyde spectrum there are four regions of energy absorption: first there is a region of very weak absorption near 400 nm (e 10^{-3}). Second, stronger but still weak bands (e 10-100) occur near 270 - 280 nm. Third, there are moderately strong bands of absorption (e 10-20 x 10^{3}) at 175 to 200 nm and fourth, strong bands (e 20,000) at 150-160 nm (Rydberg bands) which rupture the σ bonds of the molecule. The first and second regions mentioned are due to triplet - singlet transitions of the oxygen p_y lone pair to the anti-bonding orbital, $n \rightarrow \pi^*$. The third region is due to $\pi \rightarrow \pi^*$ and $n \rightarrow \sigma^*$ transitions and the last region is due to $n \rightarrow \sigma^*$ and $\sigma \rightarrow \sigma^*$. As the latter are usually inaccessible to investigation only the region above 200 nm is of value for the interpretation of spectra.

B. Spectral Absorptions of Carbonyls

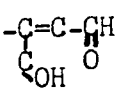
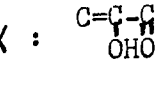
i. $n \rightarrow \pi^*$

The absorption of the carbonyl group $n \rightarrow \pi^*$ transitions in aldehydes and ketones in general is around 270-290 nm. Very weak absorptions are seen at 435 nm. A dicarbonyl, as in glyoxal (HCO-CO-H) or diacetyl (CH_3-CO-CO-CH_3) also absorbs in the region 400-500 nm due to $n \rightarrow \pi^*$ transitions of the triplet state (344). The absorbance at this low energy displays fine structure in non-polar solvents (223) (344). The $n \rightarrow \pi^*$ transition can only occur (at lower energies anyway) when the unshaired pair of electrons is definitely not involved in any bonding. If the unshaired pair becomes involved in a dative bond the characteristic long wavelength $n \rightarrow \pi^*$ absorption disappears. This is observed for long pairs on O or N in aldehydes, ketones, phenols, amines, and heterocyclic bases. In acid solutions the same loss of the $n \rightarrow \pi^*$ absorption is seen as a proton adds to the unshaired pair. In very reactive aldehydes the transition vanishes in water or alcohol solutions; hydrates of aldehydes form easily and the carbonyl nature of the aldehyde disappears $HC{=}O + H_2O \rightarrow -HC(OH)_2$. Acetaldehyde for instance is 60% hydrated at 18°C (54). Likewise in alcoholic solution hemiacetals can form and the $n \rightarrow \pi^*$ transition absorptions (280 and 450 nm) tend to disappear. Ketones however, hydrate less ($< 1\%$ for acetone) (54). Ketoaldehydes, by virtue of their two adjacent carbonyls are relatively more reactive than mono-carbonyls and the spectral study of methyl glyoxal (344) has been complicated by its great affinity for water wherein hydrates are found, by its ease of polymerization especially as polymerization can be catalysed by traces of water, and by its tendency to enolize. After ridding methyl glyoxal of all water, observed absorptions were at 440

and 280 nm in non aqueous solvents (344). These absorbances quickly decreased as water was added. If alcohol were added a slower decrease occurred. Hydration therefore imposes a serious restriction on quantification of such compounds. In addition to hydration enolization was demonstrated previously in α diketones by acetylation of the enol -OH. Acetylation could lower the maximum absorption from 284 nm to 246 nm indicating that the enol CH_2= C(OH)-CHO contributed to the 284 nm absorbance as well as carbonyl $n \rightarrow \pi^*$ transitions (344). As this is seen it would complicate the interpretation of such peaks therefore enolization will be considered in more depth.

ii. Enolization

Enolization has been observed with either α dicarbonyls R-CO-CO-R or β dicarbonyls R-CO-CH_2-CO-R. The latter show absorption at 450 nm and 280 nm. Enol tautomers of dicarbonyls (α : C=C(OH)-CO- or β: -COH=CH-CO-) exist in equilibrium with the ketones and the resultant conjugated enone which is stable in configuration, is what contributes to the absorption around 280 nm by a $\pi \rightarrow \pi^*$ charge transfer absorption. The $\pi \rightarrow \pi^*$ is stronger than the $n \rightarrow \pi^*$ at 280 nm (e=12,000 compared to e=35) (283), and the latter is masked. Changes in solvent polarity result in changes in the intensity of absorbance at 280 nm therefore there must be a shift in equilibrium between keto and enol forms. The more polar the solvent the less the intensity hence less of the enol form is permitted in polar solvents. The enol is considered therefore, to adopt the cis configuration

β :  or α : 

which favours internal hydrogen bonding and a certain amount of stability. Polar solvents would interfere with H bonding and the enol would be

discouraged resulting in a lower absorbance.

Besides the normal absorption peaks of the two carbonyls and the $\pi \rightarrow \pi^*$ charge transfer of the enone, a new band is seen with the enones anywhere up to 340 nm due to the displacement of the oxygen n electron from its position in the carbonyl π orbital. This displacement is brought about by the transfer of one electron from the ethylene π orbital to the carbonyl π^* orbital. The $\pi \rightarrow \pi^*$ transition is approximately written $C=C(OH)-CH=O \rightarrow {}^{\oplus}C-C(OH)=CH-O^{\ominus}$ and is affected in a regular way by both alkyl and electron donating or withdrawing substituents. The - OH either in α or β position is a good electron donor and facilitates the charge transfer. An electron withdrawing group on the carbonyl carbon would favour this excitation. Substituents do not, however, directly affect the $n \rightarrow \pi^*$ bands. Solvent changes complicate the interpretation of spectra due to their effect on the $\pi \rightarrow \pi^*$ transition of unlike chromophores. Polar solvents favour the transition by stabilizing the excited state which has charge polarity, yet they discourage enolization. On the other hand polar solvents cause a blue shift in the $n \rightarrow \pi^*$ absorptions band which still is displaced relatively towards the red due to the enone. Non-polar solvents shift the $n \rightarrow \pi^*$ to longer wavelengths and the $\pi \rightarrow \pi^*$ to shorter wavelengths.

The dicarbonyl group itself -CO-CO, being a conjugation of 2 like chromophores, does not incur a charge transfer between its conjugated carbonyl double bonds as is found for α,β unsaturated carbonyls or dienes. Since the carbonyl oxygens remain electronegative no excited species is favoured at low energies analogous to the one for dienes: ${}^{\oplus}C-C=C-C^{\ominus}$ where one of the atoms would have to become electron deficient: $O=C-C=O \not\rightarrow {}^{\oplus}O-C=C-O^{\ominus}$ therefore the relatively intense absorption of

ketoaldehydes or diketones around 280 and 290 nm is definitely not due to such a species of charge transfer. As mentioned above neither is it due to $n \rightarrow \pi^*$ excitations because these are displaced or are too weak. The 280 nm absorption must be solely due to the enolization of the ketone forming an enal with the aldehyde. Yet the $\pi \rightarrow \pi^*$ absorbance around 210 to 217 nm in alcohol for enals or enones shifts, on the average 39 nm to the red due to an α -OH substituent or 32 nm for a β-OH substituent (283). An enol acetate, i.e. acetylated -OH, only shifts the absorbance 6 nm (83). Theoretically, an enol aldehyde or enol ketone would absorb anywhere from 243 to 256 nm. The absorption above even this wavelength, at 280 nm for ketoaldehydes, must be due to yet another chromophore the enolate anion $\backslash C{=}C{-}O^{\ominus}$. The negative charge of the substituent has very strong ability to facilitate $\pi \rightarrow \pi^*$ charge transfer and hence lowers the energy of this excitation. The spectral shift due to such an ionization was studied with cyclic β - diketones (45). Two shoulders were seen on the absorbance curve at 256 nm and 282 nm with the latter higher in intensity at low concentration but becoming lower at high concentration. The 282 nm absorbance was therefore due to dissociated anion and increased dissociation occurred upon dilution. The concentration of the anionic species can be augmented in solution most easily by alkali hydroxide which removes the proton from -OH. In acid only the $\pi \rightarrow \pi^*$ absorption was seen, at 256 nm (45), whereas in base only the 282 nm peak, due to the enolate anion was observed. Spectra of dicarbonyls therefore are interpretable within the limits discussed above. The intense absorbance shifts in alkali are therefore a test for this grouping and have been used extensively in ascertaining the presence of unknown dicarbonyls.

iii. Ketoaldehyde Spectra

Ketoaldehydes have been found as normal decomposition products by water of the ozonides of unsaturated aldehydes (129). Among the oxidation products of fats dicarbonyls have been examined (148) which give rise to yellow colours in volatile essential oils. All the electronic spectra of these were very similar exhibiting strong bands from 275 to 425 nm in non polar solvents. Alkali greatly increased the absorbance generally in these samples.

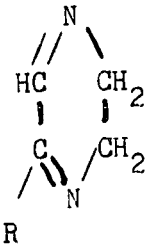
Concentrates of human urine, which showed inhibitory activity against tumors, demonstrated, in solution at pH 7 an absorbance maximum at 272 nm. At pH 1 the peak shifted to 270 nm but in alkali solution pH 10 it appeared at 292 nm (91). Such behaviour definitely suggests the presence of an enolate from a dicarbonyl, and the infrared spectrum of neutral materials confirmed the presence of both ketone and aldehyde (92)(304). Methyl glyoxal has a characteristic absorbance peak at about 272 nm shifting to broad peak centering at 290 nm in alkali. The methanol-arsenic oxide extracts in experiment 22 of this work show, with alkali, an increase in absorbance intensity at 290 nm which rose above the general absorbance in these crude extracts. The absorbance of these extracts resembles the absorbance of the fat oxidation products (148) in that there is a gradual rise in intensity from 320 nm to 220 nm showing certain distinct shoulders. It appeared from the spectra of the methanol-arsenic oxide extracts (Fig. 7) that further purification might have been necessary and therefore the methanol was driven off by heat under the influence of acid used to protect against the possible oxidation of any aldehydes to carboxylic acids. The residue, when extracted with cold ethanol, left a precipitate behind but yielded up to the ethanol a yellow colour which upon dilution showed a sharp absorbance

peak at 285 nm. The lack of a general absorption at higher wavelengths suggested at the time that the compound had been separated from much other light absorbing material. The spectral shift in alkali was not as large (only up to 293 nm) and was less intense suggesting that most of the dicarbonyl was in the enolate form before the pH was raised, or suggesting that less enolate anion could be formed after the treatment given.

The light absorbing material in solution was still able to inhibit the growth of bacteria in the same manner as did conditioned medium (Exp. 24) suggesting that the activity still correlated with ketoaldehyde-like spectral characteristics.

C. Spectra of Ethylenediamine Derivatives

To test whether the inhibitor was an α-ketoaldehyde and not a β-ketoaldehyde the reaction with ethyenediamine (EDAM) was carried out. Ethyenediamine dihydrochloride was used instead of the classical (129) O-phenylenediamine which ordinarily gives a quinoxaline derivative, since the latter reagent is unstable (308). EDAM had been used to test for the dicarbonyl compounds capable of growth inhibition in urine and clam extracts (304). Vicinal carbonyl compounds can react with EDAM to form the yellow coloured 2-substituted 5,6-dihydropyrazine compounds:



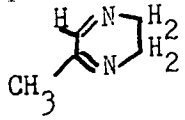
The chromophores of the yellow products are azomethine (C=N) analogues of a diene. The diimine N=C-C=N is common in pyrazine  , a polyazine. Polyazines have an absorption spectrum which depends on the position of the Nitrogens in the ring and not on the number of Nitrogens, since there is interaction between the nitrogen lone pair atomic orbitals. For example among polyazines the $n \rightarrow \pi^*$ transition band is affected by the position of the N atoms: in 1 : 2 diaza compounds (-N-N-) ground state interactions cause a red shift of the $n \rightarrow \pi^*$ band calculable from the overlap of the lone pair atomic orbitals. In 1 : 3 (-N-C-N-) and 1 : 4 (-N-C-C-N-) the interaction is weaker but pyrazine is unique (NCCN) in that the ground state interaction causes a blue shift to 327 nm of the $n \rightarrow \pi^*$ transition relative to the normal frequency which may be seen higher in dihydropyrazine for example. This occurs because no ground state overlap is allowed and any

antibonding molecular orbit for the lone pair n would have higher energy than the lowest unoccupied π orbital. Only a transition from the bonding lone pair orbital is allowed which requires more energy and therefore raises the frequency of the $n \rightarrow \pi^*$ transition (208). As the preceeding implies, a factor affecting the wavelength and intensity of $n \rightarrow \pi^*$ absorptions in azines is the percentage 's' character of the lone pair orbitals. Only the 's' component of a hybrid (s-p) lone pair orbital can contribute to the transition of an $n \rightarrow \pi^*$ since a $p_\sigma \rightarrow p_\pi$ is forbidden (209). As the 's' component of a hybrid is promoted to a π orbital the remaining σ bond orbitals of nitrogen assume a larger 's' character and thus become more stabilized. The percentage 's' character depends on the stability, therefore, on the ground state, and substituents on the chromophore affect this, and consequently the $n \rightarrow \pi^*$ transitions. This is in contrast to the lack of effect of substituents on the $n \rightarrow \pi^*$ transitions of enones.

The $n \rightarrow \pi^*$ transitions are affected by auxochromic substituents, on the carbon atoms of the chromophore, which stabilize either the ground state or the excited state through inductive effects on the N lone pair electrons. In general the excited state of an $n \rightarrow \pi^*$ transition transfers charge from the nitrogen to the carbon and any substituent on the carbon accepting electrons encourages this, consequently stabilizing the excited state relative to the ground state, and reducing the energy of such a transition. Therefore electron accepting groups (eg: C=C, O=C-OH, O=C-H, N=C-H, NH_2) cause a red shift of the $n \rightarrow \pi^*$ absorbance maximum, and conversely donors (eg: CH_3, -OH, $-OCH_3$) cause a blue shift.

In relatively non polar solvents many vibrational peaks of the $n \rightarrow \pi^*$ transition of pyrazine are seen (209). The band maximum intensity is the

band origin (V_{o-o}) but in 2-CH_3 pyrazine it is the o-1, and in 2:5 diCH_3 pyrazine it is the o-2 band. Therefore it is not easy to identify accurately the shifts of one $n \rightarrow \pi^*$ band due to substituents. In polar solvents this fine structure is blurred into one peak and shifts of the one maximum are assigned more accurately. As the product of a keto-aldehyde and EDAM contains the diimine N=C-C=N in a ring, an approximate interpretation of the spectra of such products can be made by a comparison with pyrazine derivatives.

Pyrazine exhibits the $n \rightarrow \pi^*$ band at around 327 nm, a $\pi \rightarrow \pi^*$ band at 260 nm (local excitation of the type $\overline{N\text{-}C\text{-}C\text{-}N}^{*}$) and a $\pi \rightarrow \pi^*$ band at 220 nm (charge transfer type $\overset{+}{N}\text{-}C\text{=}C\text{-}\bar{N}$) (283). 2-Methyl pyrazine shows the bands at 320 nm and 266 nm, the former shifting to 295 nm in more polar solvents. In the reaction product of methylglyoxal and EDAM the absorbance maxima in alcohol indicate three vibrational peaks which can be assigned to the $n \rightarrow \pi^*$ transitions at 405, 376 nm, and 344nm. The second band has the maximum intensity and therefore resembles the o-1 band in 2-methyl pyrazine, as indeed the product should be the 2-methyl derivative: 2-methyl dihydropyrazine  . Water as solvent blurred the V_{o-o} and V_{o-2} bands leaving the 375 nm peak as one maximum intensity (Fig. 14). That this peak represents the $n \rightarrow \pi^*$ transition is seen by its shift to lower wavelengths (340 nm) as solvent became more ionized with Na OH, and hydrogen bonding took place; the lone pair on azine nitrogen requiring more energy for its promotion to π^*. The blue shift measures the electron donating capacity and the basicity of the azine.

The band at 288 nm was not solvent-dependant as it remained static amid pH and polarity changes. It is therefore not a charge transfer

band but must be due to two conjugated like chromophores: N=C-C=N, in a local excitation. The charge transfer absorption at 228 nm was increased in intensity by the polar solvent which of course stabilized the excited state which in this case is polar.

The absorbance of the product of EDAM and the unknown ketoaldehyde from the methanol-As_2O_3 extract, displayed in acidified water two $n \rightarrow \pi^*$ absorptions at 383 nm and 328 nm (Fig. 15). These were both $n \rightarrow \pi^*$ since they both shifted to lower wavelength (332 and 297 nm) on acidification. Stronger acidification made the first band disappear but the second (297 nm) persisted. The first could be expected to disappear by cation formation with an H^+ ion but the second cannot be the same. Since there are two nitrogens in the dihydropyrazine ring the lone pair of one must in some way have been prevented from protonation more than the other. The explanation for this will appear below when the actual structure of the unknown ketoaldehyde will be discussed. The local excitation of the diimine is seen at 285 nm unaffected by solvent changes while the $\pi \rightarrow \pi^*$ charge transfer band is seen at 220 nm and of course increases in intensity in polar solvents.

After further purification of the methanol-As_2O_3 extract by heat evaporation the resultant product with EDAM gave one $n \rightarrow \pi^*$ absorption at 322 nm, a slight absorption at 275 nm indicating an altered N=C-C=N chromophore in local excitation, and high charge transfer absorption below 240 nm (Fig. 16). Upon acidification the 322 nm peak disappeared coincident with an intense absorbance at 280 nm. The bands near this wavelength which had appeared in the other spectra (at 288 nm and at 285 nm in the methylglyoxal reaction, and with the methanol-As_2O_3 extract) have been assigned generally to $\pi \rightarrow \pi^*$ local excitations.

But the chromophore absorbing at 288 nm must be different from that absorbing at 280 nm not only because of the different wavelengths but because of the appearance in acid of the latter while the 288 nm peak remained insensitive to solvent effects. The 280 nm peak must therefore be a charge transfer absorption yet not the same as the one at 228 nm for the conjugation of two like chromophores N=C=C=N. It may be due to the conjugation of two unlike chromophores. Why this derivative should differ from the first and second compounds of Figures 14 and 15 must have something to do with the purification procedure. The change to a charge transfer band of two unlike chromophores indicated a change in covalent unsaturation but the structure will be explained below after the infrared studies are considered.

D. Dinitrophenylhydrazine Derivatives

The dinitrophenylhydrazine (DNPH) derivatives of ketones and aldehydes can reveal much information on the structure of the latter. The EDAM derivatives served to indicate the two carbonyls are vicinal. DNPH reacts through its hydrazine N's with carbonyls. Water is eliminated and a C=N bond replaces C=O. Both vicinal carbonyls give a bis DNPH derivative (113). Derivatives have been studied spectroscopically and correlations of structure with spectra have been made. Unfortunately ketoaldehyde derivatives are rare so a comparison of spectra of monocarbonyl derivatives will have to suffice.

The formation of the DNPH derivative had to be carried out in the cold to obviate any reaction with sugars or other hydroxy-carbonyls which give the osazone only with heating. Since the purified ketoaldehyde from a column fractionation (Exp. 23) was used as starting material, little contaminating material was expected and indeed the derivative red crystals chromatographed as one spot on thin layer plates. The derivative of a keto-acid would have been soluble in Na_2CO_3 solution (229) with a characteristic brown colour but its absence helped indicate the purity of the sample. The crystals melted at 220°C. The formation of these crystals as an indication of the presence of a ketoaldehyde was useful for correlating the presence of a ketoaldehyde with the presence of antibacterial activity (Exp. 27) in the alcoholic extracts.

The colour of the osazone can reveal some interesting points. The 2,4-dinitrophenylosazones of sugars are yellow-brown or orange although the 4-nitrophenylosazones of sugars are all red (229). As the derivative made in these studies was from 2,4-DNPH, and was red, it would likely have a double bond conjugated with the imino linkage as this imparts the

red colour to such derivatives (260). If the derivative were yellow it would not be an α,β unsaturated carbonyl. Those DNPosazones with no double bonds conjugated with the imino linkage absorb light maximally in the region 349-368 nm. With α,β unsaturation the absorption is around 380 nm (55)(156)(157)(31). The absorption peaks of the methyl glyoxal DNPH derivative are 393 and 436 nm which both appear to be due to the two conjugated imino linkages. These absorption maxima are in agreement with those found before (24). In addition a separate absorption exists at 375 nm characteristic of saturation in the rest of the molecule (Fig.17). In comparison the unknown osazone red crystals in neutral solution had absorption maxima at 395 nm and 430 nm. The lack of absorption below 380 nm, that is from 350 to 380, definitely indicates a lack of saturation and therefore the presence of double bonds conjugated with the imino linkages.

The real value of DNPH derivatives is their absorption behaviour in alcoholic-KOH. Single carbonyl derivatives in alkali appear deep red or magenta in colour and absorb from 450 to 520 nm depending on increasing unsaturation or aromaticity. But di-carbonyls which become di-substituted with DNPH display a blue or blue-violet colour absorbing around 560 nm, for example diacetyl-bis-DNPH at 551 nm, methyl glyoxal at 561 nm, and glyoxal at 575 nm (229). The blue colour of the unknown osazone absorbed at 566 nm (Fig. 18) indicating the presence of the bis-derivative and confirming the α dicarbonyl nature of the unknown ketoaldehyde.

The infra-red spectrum of the dried red osazone (Exp. 29) is the criterion for interpreting the structure. DNPH derivatives of carbonyls have been well studied by infra red techniques and the interpretation of their spectra has been documented as a guide to assigning absorption

bands to such structures as are common in these derivatives (157) (262). The chart of N. B. Colthrup on the characteristic infra-red group frequencies was used and the table of infra-red absorptions of functional groups from 'Application of Absorption Spectroscopy of Organic Compounds' by J. R. Dyer (90). In addition tables and notes from 'Introduction to Practical Infrared Spectroscopy' by A. D. Cross (77), from 'The Infra red Spectra of Complex Organic Molecules' by L. J. Bellamy (38), and from 'Elementary Infra red Spectroscopy' by C. E. Meloan (especially for olefin absorptions) (217) were studied. Table 25 presents the assignment of chemical groupings to the spectra collected in this work.

In experiment 29 the infra-red spectrum of the red osazone indicated that certain groups were absent, namely hydroxyl, around 3400/cm; primary amine 3300/cm; aliphatic hydrocarbon C-H stretch at and below 3000/cm and the C-H deformation from 1480 to 1430/cm for CH_2 and CH_3; acid COOH at 1700/cm and ester at 1735/cm. No furan C-O at 1020/cm exists and there is an absorption between 10 and 12 microns. The absence of absorptions here in DNPH derivatives would indicate a furan (157). There is no aromatic nucleus in the carbonyl compound as it would show four strong bands in the region 769-660/cm in DNPH derivatives (157). What bands are present besides those belonging to the dinitrophenyl grouping are a C=C stretch band at 1645/cm which is the range of the vinyl group $-CH=CH_2$ absorption. A double bond with a <u>trans</u> di-substitution would absorb at 1676/cm, <u>cis</u> at 1660/cm, vinylidene at 1658/cm, tri-substituted C=C at 1678/cm and tetra-substituted at 1672/cm (217). Confirmation of the vinyl grouping is seen at 1410/cm where the in plane deformation of $=CH_2$ absorbs. The in-plane -CH= deformation at 1300/cm would be hidden by nitro group absorptions. The out-of-plane

bending vibrations at 962/cm (for -CH=) and at 922/cm (for $=CH_2$) are evident. Although other groups absorb around 920/cm the strength of this band and the presence of its overtone at 1840/cm indicate the vinyl group (217). The -CH= bending vibration in vinyl groups is usually at a higher frequency than 962/cm (at 990/cm). Indeed 962/cm is the region of absorption of -C-H bending of a trans disubstituted alkene but the latter has no absorption anywhere near 920/cm. Cis disubstituted alkenes have no absorption anywhere from 900 to 1000/cm. Therefore the 922 and 962/cm bands have to be vinyl group absorptions as predicted by the C=C stretch frequency. Because these absorptions are normally listed at 990 and 910/cm (90) they probably apply to vinyls in hydrocarbon olefins. In $\overset{\alpha}{C}-C=CH_2$ the α carbon has an effect on the band. The presence of electronegative functional groups on the α carbon shifts the absorptions (30) (290). Therefore where a vinyl is conjugated with the C=N derived from the keto group a shift must be expected. The red colour of the osazone and the electronic absorptions above 380 nm indicate that conjugation exists and the shift of the 990 and 910/cm bands to 962 and 920/cm confirms the conjugation. An example other than the red osazone is the derivative of acrylaldehyde which shows the bands at 955 and 922/cm (262). The derivative of acrylaldehyde displays a spectrum almost identical in this respect to the one produced here in Fig. 19 as the $-HC=CH_2$ is conjugated to the imino link. The C-H stretching band of hydrogen on an alkene carbon is readily distinguishable from alkane $-CH_2-$ or $-CH_3$. The vinyl C-H have bands at 3079/cm for $=CH_2$ and 3025/cm for -CH= (38). The $=CH_2$ band would be hidden in DNPH derivatives by the aromatic C-H on the DNPH absorbing at 3080/cm, but a band is seen at 3033/cm attributable to the -CH= in the red osazone.

The definite presence of the vinyl group conjugated to the imine C=N of the ketoaldehyde DNPH derivative indicates that there is no more room for further substituents, $HC^{N}\text{-}C^{N}\text{-}CH=CH_2$, indeed no alkane or other groups can be accounted for. Therefore vinyl glyoxal appears to be the parent of the red DNPH derivative from purified extracts. The hydrazone of acrylaldehyde (a mono-carbonyl derivative $HC^{N}\text{-}CH=CH_2$) is analogous to that of vinyl glyoxal (the dicarbonyl derivative) and its IR spectrum (262) is nearly identical to Fig. 19 except for the bands at 13.81 μ (always found for monocarbonyls and not for dicarbonyls), and the stretch at 9.95 μ for the C-C between the conjugated groups. In dicarbonyls the dipole moment of this bond is obviously different.

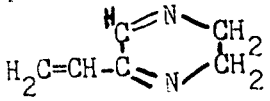
TABLE 27. Assignment of chemical groupings to absorption bands spectra of DNPH derivatives of carbonyl compounds. (in wavenumbers)

VINYL GLYOXAL	ACROLEIN	GROUP	4-OH,2-KETO BUTYRALDEHYDE	METHYL GLYOXAL
		O-H str	3450	
3290		N-H str	3280	3280
3080		C-H str aromatic	3080	3080
3033		C-H str vinyl $=\dot{C}-H$		2925
		C-H str alkane-CH_3	2860	
		C-H str methylene-$\dot{C}H_2$	2860	
1840		overtone 920		
1645		C=C str vinyl $-\dot{C}H=CH_2$		
1615	1618	C=C aromatic	1612	1613
1580	1590	C=C ar & C=N	1575	1590
1515	1538	C=C ar & N=O	1515	1525
1480	1508	N=O str	1492	1495
		C-H def. $-CH_2-$	1470(sh)	
		C-H def. $-CH_3$		1430
		C-H def. CH_2OH (electro negative)	1420	
1410	1417	C-H def. $=CH_2$		
		$-CH_3$ sym. def.		1370(sh)
1333	1325	N-- O bonded	1333	1335
1310	1307	N-- O not bonded	1310	1310
1255	1274	=N-N- str	1270	1260
1210	1217	C-H def 1,2,4-sub.arom	1220	1218
1190 sh	1195 sh	C-N	1195 w.sh	1190 w.sh
1150	1136	C-H def 1,2,4-sub. arom	1136	1140

TABLE 27 continued.

VINYL GLYOXAL	ACROLEIN	GROUP	4-OH,2-KETO BUTYRALDEHYDE	METHYL GLYOXAL
1120		C-C in cross conj.		
		C-OH	1120	
1105	1083	C-H def 1,2,4-sub.arom	1080	1086
1060	1062		1050	1055
		C-O str	1036	
		C-C str ($C-CH_3$)		1019
		(C-C at 1000 cm^{-1} in 4-OHbutyraldehyde DNPH) $-N=C-CH_2CH_2CH_2OH$		
962	957	C-H def. C-H, CH_2		
845	835	doublet, 2 free C-H arom. def.	840	842
830	830		830	833
762	765	skeletal vibration not all long, therefore not aromatic.	760	760
742	743		740	742
705		no 725 band therefore not mono derivative.		
692	683		683	685
	724	skeletal vibr. of mono.deriv.		
		(skeletal vibr. of 4-OH butyraldehyde includes 722 band)		

E. Reexamination of EDAM Derivatives

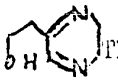
It can be deduced that the ethylene diamine derivatives of the purified ketoaldehyde (Fig. 16) must have been 2-vinyl-5,6-dihydropyrazine  . This compound contained the enimine chromophore CH_2=CH-C=N which in its simplest form absorbs around 220 nm for a $\pi \rightarrow \pi$ charge transfer. The enimeme is insensitive to alkyl substituents (53) therefore the other -C=N has no effect on the absorbance. The $n \rightarrow \pi^*$ excitation is visible around 322 nm but on acidification it disappeared because the lone pair bound a proton becoming the charged species $C=C-C=\overset{+}{N}H-$ for which the $\pi \rightarrow \pi$ band shifted 60 nm to the red (283) to absorb at 280 nm. Under alkaline conditions the proton was removed and the 280 nm band disappeared because the charged chromophore existed no longer (Fig. 16). Coincidentally the $n \rightarrow \pi^*$ reappeared at 322 nm in the alkaline solution. The red shift and the hyperchromic effect of the $\pi \rightarrow \pi^*$ transition in acid is due to a facilitated $\pi \rightarrow \pi^*$ charge transfer because the protonation of the lone pair $C=\underset{\curvearrowright}{C}-\underset{\curvearrowright}{C}=\overset{+}{N}H-$ enhances the electron withdrawing capacity of the auxochrome. That this type of transfer explains the observed spectral behaviour is further proof that the vinyl is conjugated to the ketone of the ketoaldehyde. The vinyl substituted compound after EDAM reaction was not made from the methanol-As_2O_3 extracts but from the purified material after heat evaporation of the alcohols used as solvents. As the EDAM derivatives differed in their chromophore absorptions, a difference in their covalent saturation was indicated. This suggested the vinyl group might not be present in the methanol-As_2O_3 extracts. Therefore, as a precaution against the effects of heating the extracts, they were bubbled with nitrogen gas to evaporate the alcohols during the purification process (Exp. 31). The

The derivative DNPH crystals melted at 245°C. They were yellow-orange not red, an immediate indication that saturation existed between the two carbons formerly recognized as part of the vinyl group. Saturation instead of unsaturation explains the difference in the spectra of the EDAM derivatives (Fig. 15). In the saturated form no enimene chromophore C=C-C=N existed, and therefore no consequent facilitated $\pi \rightarrow \pi^*$ charge transfer band could occur on acidification.

The absence of unsaturation in the yellow-orange osazone was confirmed by the IR spectrum (Fig. 21). There is no C=C stretch band at 1645/cm and no -CH= deformation at 960. There remained only a weak absorption around 920/cm, of the groups other than $=CH_2$, and no overtone at 1840/cm was visible. The $=CH_2$ in-plane deformation band (1410/cm) was missing and a band at 1420/cm was found. It can be assigned to a methylene $-CH_2-$ group in a very electronegative environment (217), shifted from its normal position at 1470/cm. At 1470/cm there appears a shoulder peak accounting for a normal aliphatic $-CH_2-$ group. There is no peak from 1430/cm to 1470/cm so it is likely that no $-CH_3$ is present (217). Stretch frequencies of $-CH_2$ groups absorb at 2860/cm and an absorption here in the spectrum of this osazone confirms the presence of aliphatic C-H. There is a hydroxyl group band at 3450/cm and a weak C-O stretch band as a shoulder at 1036/cm. The hydroxyl must be responsible for the electronegative environment on one of the $-CH_2-$ groups and since there is no methyl group the $-CH_2-OH$ group must be at the end of the chain $HC(=N)-C(=N)-CH_2-CH_2OH$. The bis-DNPH derivative of 4-hydroxy, 2-ketobutyraldehyde has an IR spectrum almost identical to its analogue the osazone of deoxy-D-glucosulose (113) which has only two more hydroxymethyl groups. Consequently the IR spectrum of the latter only has a broader O-H stretch band. As the 4-hydroxyl

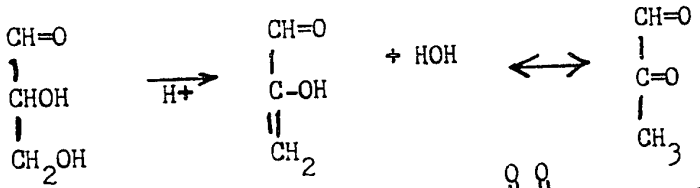
group is in a position β to the ketone in the ketoaldehyde it can be removed by β elimination due to heating, especially in acid conditions as these methanol-As_2O_3 extracts were. The β elimination process dehydrates the molecule leaving α,β unsaturated aldehydes or ketones, in fact the latter are commonly formed by the acid or base catalysed dehydration of β hydroxy aldehydes or ketones (230). Both the acid and base catalysed dehydration are called β eliminations. The carbonyl group is polar with an excess of charge on the oxygen and a deficiency on the carbon. The carbonyl is a good acceptor and hence attracts electrons from the α carbon. As electrons shift to the carbonyl the α hydrogens become less strongly bound and their acidity increases, $H\ O\ H^{+}\ -$ $C-C\rightarrow C=O$. The hydrogen ion leaves and takes the β-OH group, hence the double bond forms between the α and β carbons (230). β hydroxy carbonyls lose water readily because the resulting α,β unsaturated aldehyde or ketone is conjugated with the carbonyl group and conjugated systems are more stable than non conjugated systems. If a hydroxyl group is on the carbon α to the carbonyl group it is more tightly held than when it is on β or any other aliphatic carbon $\overset{O}{\underset{|}{-C}}\rightarrow C=O$ and it is certainly not capable of being eliminated by heating (230). Experiment 32 of this work shows that heating the extracts in acid dehydrated the molecule to give the vinyl form, while cooling maintained the position of the hydroxy group intact. Therefore because elimination is a process observed in these experiments there is little question as to the position of the -OH group in the ketoaldehyde isolated in methanol-As_2O_3 extracts: it is most probably on the β carbon and not the α carbon.

The ketoaldehyde from the original extracts is 4-hydroxy-2-ketobutyraldehyde. With EDAM such a compound would produce 2-hydroxyethyl-5,

6-dihydropyrazine and consequently the hydroxyl would form an internal hydrogen bond with the N lone pair at position 1:  This accounts for a difference in $n \rightarrow \pi^*$ transitions of the lone pairs on the two nitrogens at 383 nm and 328 nm (Fig. 15). Both are shifted to the blue (332 and 297 nm) as the polarity and acidity of the solvent is increased but the one $n \rightarrow \pi^*$ transition on position 4 is obliterated first by acidification while position 1 n electrons($n \rightarrow \pi$ 297 nm) avoid protonation because of their involvement with the hydroxyl hydrogen bond, until stronger acid competes strongly enough. The chromophore $CH_2=CH-$  , 2-vinyl-5, 6-dihydropyrazine, in acid, absorbs at 280 nm because of the charged enimine, but in alkali the charge is removed and the $n \rightarrow \pi$ transition is restored, and absorbs at 322 nm. As the alkaline solution was polar the n electrons were as strongly hydrogen-bound to the solvent as possible yet the absorption (at 322 nm) was still at higher wavelength than the same strongly bound lone pair in 2-hydroxyethyl-5, 6-dihydropyrazine (at 297 nm). The difference between the two compounds was that the vinyl group is an electron accepting group and the hydroxyethyl is an electron donating group. The latter stabilizes the ground state while the former stabilizes the excited state, which reduces the energy of transition and shifts the $n \rightarrow \pi^*$ to higher wavelengths. Such behaviour satisfactorily explains the spectra for these compounds.

F. Dehydration

On carbonyl compounds the α position would hold the -OH group strongly while a β -OH group would be readily eliminated. Glyceraldehyde and dihydroxy acetone are believed to be inter-convertible metabolically and glyceraldehyde can dehydrate through acid catalysis and heat to give methylglyoxal. The α oxygen remains while the β -OH is eliminated (230). This is one method of producing a ketoaldehyde and it occurs readily because a conjugated system

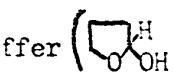
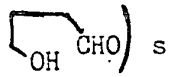
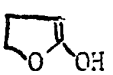


is derived. Dipole moment measurements show C-C is flat which corresponds with a restrained rotation of the C-C bond (44). When the system is planar, electronic interaction, resulting from mutual overlap of the orbitals, is at a maximum and the difference in energy levels between the ground state and the excited state is decreased. The readiness of this type of dehydration is emphasized during heating of the reaction with DNPH in acid, of not only dialdehydes and diketones but with isonitroso-ketones, dioximes, α-hydroxyaldehydes and α-halogenoketones (129) all which give bis-DNPH derivatives. Many ketoalcohols form ketoaldehydes by heated oxidation in the presence of metal salts (129) and it has been recently shown (257) that methyl glyoxal occurs in tissues from the dehydration of dihydroxyacetone or glyceraldehyde catalysed by polyvalent anions. Phosphate, bicarbonate, and especially phosphorylated sugars like glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and fructose-1, 6-diphosphate ($F_{1,}$6 diP) all catalysed methyl glyoxal formation. Pyrophosphate and ATP did not but these are polyphosphates.

These methods of ketoaldehyde formation are not well controllable and in order to choose the best method of synthesis of hydroxyethyl glyoxal an expert in the synthesis of ketoaldehydes was consulted.

G. Synthesis of Ketoaldehydes

Dr. Gabor Fodor of Laval University suggested the two step reaction used in these studies. The synthesis of 4-OH, 2-ketobutyraldehyde from γ-butyrolactone was carried out and about 1 ml was recovered. The hydroxyaldehyde had the density, I.R. absorption spectrum, and DNPH derivative melting point of the 4-OH, butyraldehyde studied by Paul (241).

The I.R. spectrum of the hemiacetal or hydroxyaldehyde would not differ ( or ) skeletally from that of the lactone  but a few differences were notable: the lactone could enolize very slightly  and its I.R. absorption showed a small contribution from an OH group at 3500/cm showing internal hydrogen bonding, and less at 3600/cm showing free OH. Predominantly the lactone carbonyl gives a strong band from 1760/cm to 1800/cm; a very weak contribution of the C=C bond from the enol form is present at 1660/cm. The C-O of the hydroxyl C-OH is not apparent in the lactone presumably due to the small quantity of the enol form but C-OH absorption appeared in the hydroxyaldehyde at 1036/cm along with a consequent reduction in absorption of the C-O-C band (at 1180/cm) due to the hemiacetal's ability to open the ring and free the -OH and HC=O groups. In the hydroxyaldehyde, the -OH absorbs at 3380/cm and the C=O absorption of aldehyde is at a lower frequency than lactone and is found at 1750/cm. The rest of the hydroxyaldehyde spectrum is similar to the lactone in its skeletal aliphatic nature.

The oxidation of a monocarbonyl with selenium dioxide selectively adds oxygen to the α carbon producing an α dicarbonyl (259), therefore the 4-OH butyraldehyde produced 4-OH, 2-ketobutyraldehyde under this treatment. The synthetic product, a viscous brown liquid, had the smell of rotting apples as noted in the early isolation of the natural

substance (Exp.20). The I.R. spectrum was recorded and the -OH group appeared to absorb from 3400 to 3500/cm. Two carbonyl bands are seen in the infrared at 1760 and 1725/cm. These free carbonyls would suggest, as would the free aldehyde in the hydroxyaldehyde, that the open chain forms rather than cyclic hemiacetals are preferred.

The DNPH derivative of the synthetic ketoaldehyde was made and yellow-orange crystals formed. They melted at 246°C comparable to 245°C for the isolated natural derivative (Exp. 32). By heating a sample of the synthetic ketoaldehyde in acid a product was formed which made a derivative with DNPH of red crystals. They melted at 221°C compared to 220°C for the derivative from active extracts. The I.R. spectra of both these osazones matched exactly the appropriate spectra from the natural products and this identity is sufficiently good that the ketoaldehyde isolated from material inhibitory for cell growth, is likely 4-OH, 2-ketobutyraldehyde.

H. Explanation of Electronic Spectra

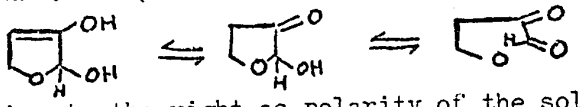
i. Synthetic Hydroxyethylglyoxal

The synthetic 4-OH, 2-ketobutyraldehyde was submitted to electronic spectral studies. When dissolved in hexane (Fig. 27) an absorption curve was obtained rising from 320 nm to a plateau (300 to 260 nm) and further rising from 260 nm to stronger absorption around 212 nm; a shoulder appeared at 250 nm and a small peak appeared as a bump at 228 nm. In chloroform the same general pattern appeared; the stronger high frequency absorption rose below 230 nm but was not seen in this solvent, while the bump shifted to 240 nm. A slight rise in absorbance was seen at 285 nm in this more polar solvent. In ethanol the increasing absorption at lower wavelength rose to a peak at 209 nm with a few weak shoulders at 222 nm and 228 nm. The high frequency peak appeared at 201 nm in water. This spectral display on increasing solvent polarity is susceptible to the following interpretation. The peak at 212 nm in hexane, which shifted to lower wavelength (201 nm) with a higher extinction in water, is likely due to an excitation of an n lone pair electron. At this wavelength, it is probably an $n \rightarrow \sigma^*$ (283) on the carbonyl $\rangle C = \ddot{O} \rightarrow \rangle C \underline{\sigma^*} \dot{O}$, for $n \rightarrow \pi^*$ usually absorbs at a lower energy, around 285 nm. The $n \rightarrow \pi^*$ of carbonyl groups was seen faintly at 280 nm and 310 nm (displaced $n \rightarrow \pi^*$) in the polar solvents chloroform and ethanol but its absence in hexane and the relatively low extinction of $n \rightarrow \sigma^*$ in hexane indicated a paucity of free carbonyl groups in this non polar solvent. The appearance of the 285 and 310 bands, and the rise in the 210 nm absorption in more polar solvents (chloroform and ethanol), and the appearance of shoulders at 222 and 228 nm (very possibly other vibrational peaks of $n \rightarrow \sigma^*$) indicate an increase in amount of free carbonyl groups.

Water, however, reduced the shoulders at 222 and 228 nm (ν_{o-o}, ν_{o-l}) suggesting strongly that they are indeed both due to n lone pair electrons on carbonyl oxygen and hydrogen bonding took place between the lone pair and the water.

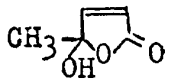
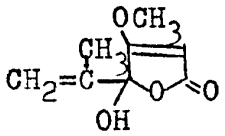
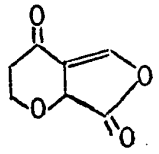
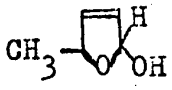
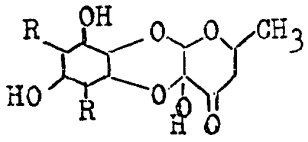
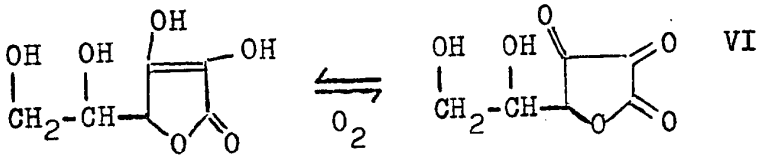
The bump on the curve at 228 nm in hexane and at 240 nm in polar solvents must be due to a $\pi \rightarrow \pi^*$ charge transfer absorption as it moves to lower energy with increasing solvent polarity. Such a chromophore may be due to an enol $\diagdown C{=}C \diagup^{OH}$ forming an α-OH enal $\diagdown C{=}\overset{OH}{\overset{|}{C}}\text{-CHO}$ conjugation with the aldehyde, but theoretically this should absorb around 255 nm in ethanol, and not around 240 nm. Otherwise a $\pi \rightarrow \pi^*$ charge transfer does not exist in hydroxyethylglyoxal, therefore it could come from the dehydrated compound vinyl glyoxal $CH_2 = CH\text{-}\overset{O}{\overset{\|}{C}}\text{-CHO}$ present as a contaminant in small amounts. The 228 nm $\rightarrow$ 240 nm shift from hexane to ethanol could definitely indicate an enone absorption, as 11 nm is the value for correcting enone absorption maximum in hexane against ethanol (283).

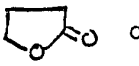
ii. Cyclization

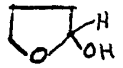
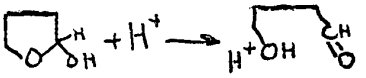
The enol tautomer is favoured in non polar solvents (283) (45). A lack of free carbonyl group absorptions was evident in such solvents with hydroxyethyl glyoxal. A cyclic enol form of hydroxyethyl glyoxal is therefore suggested  with equilibrium shifting to the right as polarity of the solvent increases. This is thoroughly compatible with the spectral data discussed above.

The cyclic form of related compounds eg: protoanemonin (I Table 28) (289), Penicillinic acid (II Table 28) (289) (251) (252), patulin (III Table 28) (41), 4-hydroxy-2-pentenal (hemiacetal) (IV Table 28) (275), actinospectacin (V Table 28) (341), and ascorbic acid (VI Table 28) all of which

Table 28 Structures of some carbonyl compounds

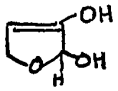
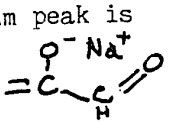
Protoanemonin		I
Penicillinic Acid		II
Patulin		III
4-Hydroxy-2-pentenal		IV
Actinospectacin		V
Ascorbic Acid		VI

are antibiotics or growth inhibitors, is stable at physiological pH, with electronic spectra displaying no absorbance due to the carbonyls involved in ring formation. Alkali (NaOH) can open the rings however and a rise in absorbance due to the freeing of carbonyls is seen around 280 nm (280) (251). The opening of the ring with alkali is commonly used in work with such structures as penicillinic acid (252), aglycones of the cardiac glycosides (eg: ouabain) (199) (264), and angelica lactone (152) but that is because these lactones  or pseudo acids  form salts of carboxylic acids with alkalis.

A hemiacetal however  , like a hydrated aldehyde, is dissociated also under acidic conditions (230)  and when HCl was added to hydroxyethyl glyoxal in ethanol a rise in absorption around 280 nm and 310 nm was seen (Fig. 28) interpretable as the freeing of carbonyl groups by the opening of the ring. Although the $n \rightarrow \pi^*$ bands are displaced, acid lowers them due to hydrogen bonding. Acid, by freeing the carbonyls from the hydrated form in water was reported to increase the absorbance of methyl glyoxal at 270 nm (304). Alkali (NaOH) as expected, affected the spectrum of hydroxyethyl glyoxal by raising the absorption to around 290-310 nm (Fig. 28), believed to be due to formation of conjugated enolate anions and the displaced $n \rightarrow \pi^*$ band as discussed earlier. When the infrared spectrum of hydroxyethyl glyoxal was taken a thin film of the liquid was placed between two NaCl blocks. The dicarbonyl grouping was seen in this case. In pure liquid what exists, therefore appears to be the more polar free carbonyl configuration whereas in alcohol (in U.V. studies) there may be, besides a cyclic hemiacetal, hemiacetal formation of the aldehyde with the solvent itself. Such a difference in what the two spectra indicated was noted as

an apparent discrepancy by Shaw (289) and Raphael (251) with penicillinic acid, where the I.R. showed an open chain form and the electronic absorptions showed cyclic structures.

iii. Extracts of Natural Hydroxyethylglyoxal

The similarity of the spectra of synthethic hydroxyethylglyoxal to those of the methanol-As_2O_3 extracts of actively inhibitory conditioned culture medium is very close. Some of the methanol-As_2O_3 extract was added to cyclohexane and the absorption spectra (Fig. 29) indicated a sharp peak at 212 nm, a peak appearing as a prominent shoulder at 244 nm and a plateau from 280 to 290 nm dropping at 300 nm (displaced $n \rightarrow \pi^*$ absorption). In this non polar solvent the enol would be favoured and it is seen in the 244 nm absorption which, in ethanol would be and is at 255 nm, the theoretical position of the conjugated hydroxy enal. Slight absorption at 212 nm and 300 nm indicate little free carbonyl when compared to the spectrum in methanol of methanol-As_2O_3 extracts where, for the same concentration of solute the peak at 210 nm for $n \rightarrow \sigma^*$ is 1.5 times as high as the 212 nm peak in cyclohexane, and the 280 nm absorbance rises considerably. These increases suggest more free carbonyl groups, and are compatible with the  equilibrium shift to the right as solvent polarity increases. In alcohol the 255 nm absorption for the hydroxyenal is rather weak also suggesting more of the tautomer exists. NaOH when added to the alcohol extracts raised the absorbance around 295 nm and up to 340 nm. The 295 nm peak is characteristic of the conjugated enolate anion  presumably forming a sodium salt and the 320 nm to 340 nm absorption is attributable to displaced $n \rightarrow \pi^*$ absorptions of the free carbonyls.

iv. Cation Effects

The effect of acid on the electronic spectra of hydroxyethyl glyoxal was to raise the absorbance near 280 nm. The possibility that acid opens a hemiacetal arrangement, liberating a carbonyl could account for the absorption, especially if the enol tautomer were at large as is possible in a polar environment. Acid is known to dissociate hemiacetals readily and hydrolyse acetals fairly easily. Alkalis, on the other hand do not catalyse acetal hydrolysis but would allow hemiacetals to dissociate encouraging the enolate anion to materialize. The enolate anion absorbs closer to 295 nm. The charge transfer in this type of $\pi \rightarrow \pi^*$ transition is facilitated by the electron donating effect of the ionized hydroxy group $-O^{\ominus}$. With the hemiacetal of hydroxyethyl glyoxal in ethanol NaOH raised the absorption around 295 nm and 315 nm, the latter perhaps due to the aldehyde carbonyl.

v. Synthetic Vinyl Glyoxal

The dehydration of hydroxyethylglyoxal results in a change in electronic spectra. An α,β unsaturated ketone $CH_2=\overset{|}{C}-\overset{\overset{O}{\|}}{C}-CH=O$ is the main chromophore and no ring can exist. As a result both carbonyls are free and can contribute fully to $n \rightarrow \pi^*$ absorptions. This explains why the absorption of the extracts (Fig. 13) is so high and sharp at 285 nm. Since an α,β unsaturated ketone, when excited, involves a charge transfer from the vinyl to the ketone carbonyl $C=C-\overset{\overset{O}{\|}}{C}-C=O$, it would be enhanced by a strong electron withdrawing group on the carbonyl carbon. The aldehyde carbonyl is such a group; therefore the normal enone absorption of 215 nm is shifted to 240 nm by the adjacent aldehyde. This shift is accounted for in both synthetic vinyl glyoxal (Fig. 30) (ie: hydroxyethylglyoxal heated in acidic

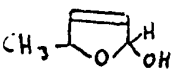
solution) and in the ethanol extracts (after heating the acidified methanol-As_2O_3 extract, (Fig. 13). Absorption at 240 nm in the alcohol by the enone also explains the bump in the absorbance spectra of synthetic hydroxyethylglyoxal, thought to be due to a contaminating amount of enone (discussed above). With alkali vinylglyoxal cannot form an enol as readily as hydroxyethylglyoxal because of the resultant cumulated diene $CH_2=C=\overset{OH}{C}-CHO$. Equilibrium does, however allow this to exist to a small degree and a shift to 297 nm for the α-OH enal is seen simultaneously with a rise in absorption at 228 nm characteristic of allenes C=C=C, ketenes C=C=O, or carbodiimides N=C=N (283). This would explain why the absorption in alkali (Fig. 13) of the ethanol extracts after heating, gave less of a rise at 297 nm than the corresponding methanol-As_2O_3 extracts; less enol and therefore less enolate ion could form in spite of the alkaline conditions.

I. Action of the Ketoaldehydes

i. Structural Implications

Hydroxyethyl glyoxal is a novel compound and its structure has not been recorded in Beilstein since 1893. In a ketoaldehyde, when both carbonyl groups are free, the grouping is slightly polar, with oxygen carrying a surplus of charge and the carbons becoming positive. Two of these electron-withdrawing groups adjacent to each other give rise to a strong electrophilic center and it is this which makes the ketoaldehyde particularly reactive. Single ketones or aldehydes are not as reactive as they have half the electrophilic capacity. This structural difference and chemical reactivity is correlated with growth inhibitory activity towards a number of cells. There are numerous reports on the cytostatic effects of aldehydes and ketones but they have a short lived action on cell division while α-ketoaldehydes have a strong effect (93). A similar electrophilic capacity can be achieved by conjugating any two electron-withdrawing groups as in an α,β unsaturated aldehyde $C{=}C{-}\overset{H}{\underset{|}{C}}{=}O$ but such compounds are indiscriminately more reactive than dicarbonyls and are usually quite generally more toxic to living cells. Acrolein for instance is believed to be the cytotoxic agent responsible for inhibitory action of spermine and spermidine, as acrolein is an oxidation product of these polyamines (1) (2).

Among the oxidation products of fats, certain unsaturated aldehydes were found (275) and some in particular had marked activity against tumor cells (277) (101) (102). It was the presence of a hydroxy group on the γ carbon of 2-pentenal $CH_3\text{-}\overset{OH}{\underset{|}{C}}H\text{-}CH{=}CH\text{-}\overset{H}{\underset{|}{C}}{=}O$ which gave this compound the activity reported, for 2-pentenal $CH_3\text{-}CH{=}CH\text{-}\overset{H}{\underset{|}{C}}{=}O$ alone had no such biological activity. In this case the only chemical

function the -OH group could perform to change the activity of 2-pentenal would be to create a hemiacetal ring structure  The importance of the γ-OH group cannot be underestimated here and the ring structure could be essential to the specific inhibitory activity of this compound over that of 2-pentenal. In the hemiacetal configuration, the carbonyl group does not exist and its electron-withdrawing effect is absent; therefore the reactivity of the whole molecule is lessened and it is not likely to react with every nucleophilic group it encounters. But should the molecule be in an environment where the ring opens (perhaps due to ionic changes) the full reactivity could then materialize. Hydroxypentenal is a more potent mitotic inhibitor than methylglyoxal or kethoxal (274) and the action of the inhibitor reported in this thesis seemed to be more potent or permanent than methylglyoxal or kethoxal against bacterial growth. These latter compounds do not contain a 4-OH group and the new ketoaldehyde does. Again the 4-OH group can provide this molecule with the alternative of being in a reactive form with 2 free carbonyls, or in a less reactive form the hemiacetal enol, with no free carbonyls. Such a compound could have remarkably subtle and specific inhibitory properties, with the equilibrium between closed and open forms dependant on such simple conditions as cation concentrations. In situ the aldehyde could even be stored in a hemiacetal linkage with an -OH group on a large molecule, and could be released as required by ionic changes; or stored in acetal linkage (glycosidic linkage); indeed the natural material was isolated from a carrier of larger size.

The inhibitory action of ketoaldehydes is reversed by sulfhydryl compounds (96). In particular α-bis-SH groups are much more

reactive than β-bis -SH, and γ-bis- SH compounds are unreactive (94). Cysteine $HS-CH_2-CH(COOH)-NH_2$ is highly reactive and so vicinal -SH and -NH groups may be equally as important as 2 vicinal -SH groups. However cysteamine $SH-CH_2CH_2-NH_2$ does not reverse ketoaldehyde inhibition (94) therefore the carboxylic acid group of cysteine plays an important part in the biological reactivity with ketoaldehydes, and this specificity of structure testifies to the unique nature of the growth control mechanism which is the target of ketoaldehyde inhibition. Glutathione (GSH) although an -SH compound, does not reverse the inhibition by ketoaldehydes (94) but it does react with them to form a thioester in the presence of glyoxalase I, whereas cysteine does not. Again another specific reaction of ketoaldehydes with an -SH compound is measurable without interference by other -SH compounds.

The action of GSH with hydroxypentenal (275) is to add the -SH across the double bond followed by cyclization. Blocking of protein -SH groups by hydroxypentenal is reversible by cysteine and GSH demonstrating the similarity between the enal and ketal with regard to their general anti-sulfhydryl behaviour. Ketoaldehydes (127) and 4-hydroxypentenal (274) both can inhibit rapidly dividing mammalian cells. The latter study indicated also that non-dividing cells were not damaged. Damage was easily assessed microscopically as cytomorphological changes in the cells. Bulges on the membranes appeared and broke off in the presence of hydroxypentenal (275) (254). They are probably due to the inhibition of certain membrane -SH groups since mercaptans too caused the same effect by swelling the cells (37).

The action of hydroxyethylglyoxal on L5178Y (Exp. 38) was also to cause some cells to swell and burst, and so its action may be presumed

to affect membrane -SH groups. The L5178Y in these studies, the Ehrlich's Ascites cells of Schauenstein (277), the many malignant types of Belkin (37), and the rapidly proliferating cells of Scaife (273) (274) all showed cytomorphological damage due to hydroxyketoaldehyde, hydroxypentenal, or mercaptans. In contrast, normal cells from solid tissues failed to produce blebs (37) and non dividing cells (274) were not damaged by these agents. This can be taken to indicate that anti-SH compounds particularly inhibit the growth of dividing cells, have less effect on non dividing cells, and at least affect the membrane of the cells that are inhibited. There is good reason to believe therefore that the natural growth-controlling substance found in these studies would be a suitable anticancer agent with little effect on other than cancer cells, since evidence has already been given that methylglyoxal, even in large doses, arrested mouse tumors while its only gross or microscopic abnormality was a suppression of testicular cell division (13) (14). It remains however to find out how the ketoaldehyde inhibits cell division.

SUMMARY

1. Medium used by HeLa cells was found to inhibit bacterial growth.

2. Virulent strains of bacteria were found to be more resistant to inhibition by culture conditioned medium (CCM) than were strains of lesser virulence.

3. HeLa cells produced the inhibitory activity in three different media.

4. Inhibitory activity was found in medium which had supported the growth of HeLa cells, human embryonic kidney cells, human embryonic lung cells, human embryonic intestine cells and human kidney cells.

5. Inhibitory activity was not found in medium which had supported the growth of Rhesus monkey kidney cells, African green monkey kidney cells, HeLa cells carrying PPLO infections or human Amnion FL cells with PPLO.

6. Inhibitory activity was stable to pH changes and temperature changes, and it was dialysable.

7. Purification of inhibitory activity by Sephadex gel filtration indicated it behaved as having a molecular weight of about 1000.

8. Cysteine appeared to reverse the inhibitory activity.

9. Methyl glyoxal appeared to show inhibitory activity similar to that of the CCM.

10. Inhibitory activity could be separated from material of 1000 mw by arsenolysis.

11. Electronic spectra of this separated activity in methanol

suggested the possible presence of carbonyl groups.

12. Preparations were transferred to ethanol after evaporation of the methanol.

13. Arsenolysis of the material from CCM without inhibitory activity (from PPLO infected cultures) showed no evidence of creating inhibitory activity or light absorbing artifacts.

14. Ethyenediamine reacted positively with a solution of separated inhibitory material by forming a bright yellow solution, suggestive of reaction with α-ketoaldehydes.

15. Dinitrophenylhydrazine (DNPH) reacted positively with ethanolic solutions of separated inhibitory material by giving red crystalline products.

16. The DNPH derivative red crystals displayed electronic spectra, similar to those of an osazone of a ketoaldehyde, by giving a positive Neuberg test.

17. Infrared spectroscopy of the red crystals suggested the ketoaldehyde had a vinyl group in conjugation with the ketone, possibly vinyl glyoxal.

18. An inhibitor from human kidney cells, prepared in a similar fashion, gave red crystals with an I.R. spectrum identical to that of the HeLa cell derivative.

19. By preparing the alcoholic extracts of active material without heating, the osazones that were derived were orange instead of red.

20. The infrared spectrum of the orange osazone suggested that a

hydroxyl group was present and the vinyl group was absent.

21. Dehydration of a β-hydroxyketone by heating was tested as being possibly responsible for the origin of the vinyl group. The possibility was borne out.

22. The structure of the parent ketoaldehyde was tentatively taken to be 4-hydroxy 2-ketobutyraldehyde, and its dehydrated offspring was taken to be vinyl glyoxal.

23. 4-hydroxybutyraldehyde was synthesized from γ-butyrolactone by an $LiAlH_4$ reduction at -35°C. I.R. spectra of the aldehyde and of the DNPH derivative, and the mp of the latter confirmed its identity.

24. A selenium dioxide oxidation of the hydroxybutyraldehyde was performed, and 4-hydroxybutyraldehyde with a 2-keto group was derived. I.R. spectra of its osazone and its dehydrated osazone matched those of material from natural sources.

25. Hydroxyethyl glyoxal was found to be a substrate for the enzyme glyoxalase I.

26. While testing bacteria it was found that their inhibited growth response, towards increasing doses of synthetic ketoaldehyde, was similar to the inhibited bacterial growth responses in the presence of the naturally derived inhibitor, at comparable concentrations.

27. Growth of the mouse lymphoblast cells L5178Y <u>in vitro</u>, was inhibited by the active material derived from human cell cultures.

28. The compound hydroxyethyl glyoxal appears to be a novel compound,

as it has not been reported in Chem. Abstracts or in Beilstein since 1893.

In conclusion structural identity of 4-hydroxy-2-ketobutyraldehyde with the ketoaldehyde produced from an active inhibitory preparation from HeLa cell medium, was tentatively assumed. The relationship of this compound to the material of larger molecular weight with which it was isolated, possibly a carrier, remains to be elucidated.

POSSIBLE FURTHER RESEARCH

1. To achieve a more precise assessment of the structure of the osazones, and therefore presumably of the carbonyl compounds, the osazones could be produced from material from CCM in considerably large quantities and subjected to elemental analysis, mass spectrography, nuclear magnetic resonance techniques, and mixed melting points.

2. Different methods of synthesizing 4-OH,ketobutyraldehyde could be attempted, to find a simpler one and one giving greater yields. For example the theoretical synthesis suggested by Prof. M. Kates appears interesting. It involves the half esterification of malic acid with ethanol to produce Et-O-CO-CH_2-CH(OH)-COOH. Refluxing this ester with $LiAlH_4$ should produce HO-CH_2-CH_2-CH(OH)-COOH which can undergo internal esterification to produce a lactone. Heating the 2 hydroxy lactone with lead acetate in benzene could produce the 2 keto derivative, which could be reduced by $LiAlH_4$ at -30°C to give the hemiacetal, itself 4-OH, 2-ketobutyraldehyde.

3. The nature of the larger inhibitory material could be examined more closely. Attempts to remove the ketoaldehyde in a variety of ways, from its carrier, may help elucidate how it is bound and what the carrier is. This should determine whether the ketoaldehyde is present as such on the carrier before it is derived experimentally, and therefore whether the natural material inhibits by means of a ketoaldehyde. Why similar fractions from PPLO infected cell medium show no activity nor chemical content capable of producing a ketoaldehyde could be investigated in comparison to the natural material to show in the latter what is present that is absent in the former.

BIBLIOGRAPHY

(1) ALARCON, R.A. (1968);
Fluorometric determination of acrolein and related compounds with m-aminophenol.
Anal. Chem. 40: 1704.

(2) ALARCON, R.A. (1969);
Acrolein detection during the enzymatic oxidation of spermine or spermidine.
Fed. Proc. 28: 805.

(3) ALLEN, M.J. (1966);
Electrochemical aspects of metabolism in Symposium on Bioelectrochemistry of Microorganisms.
Bacteriol. Rev. 30: 80.

(4) ALLFREY, V.G., R. FAULKNER & A.E. MIRSKY. (1964);
Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis.
Proc. Nat. Acad. Sci. (U.S.) 51: 786.

(5) ALLON, R.J.F., W. BROWN, & A.C. SCOTT (1971);
Infections caused by meningococci of Serogroup Z.
The Lancet #7703 1: 783.

(6) ALTUCCI, P., & G. TARRO (1964);
Sull'azione inattivante in vitro di alcuni derivati chetoaldeidici nei riguardi degli arbovirus.
Bolletino della Soc. Ital. di Biol. Speriment. XL: 1224.

(7) AMERSBACH, J.C., E.M. WALTER & E.S. COOK (1942);
Respiration of human keratoses and epitheliomas.
Arch. Dermat. & Syph. 46: 269.

(8) AMERSBACH, J.C., E.M. WALTER & G.S. SPERTI (1946);
Treatment of basal cell epithelioma by injection of tissue extracts.
Arch. Derm. Syph. 54: 119.

(9) AMERSBACH, J.C., E.M. WALTER & G.S. SPERTI (1950);
Treatment of human malignancies with tissue extracts.
Acta. Unio Intern. Contra Cancrum 6: 1073.

(10) ANET, E.F.L.J. (1962);
Degradation of Carbohydrates. I. Isolation of 3-deoxyhexoses.
Aust. J. Chem. 13: 396.

(11) ANET, E.F.L.J. & K. MICKLE (1959);
Chemistry of non-enzymic browning. VII. Crystalline di-D-Fructose-Glycine and some related compounds.
Aust. J. Chem. 12: 280.

(12) APPLE, M.A. & D.M. GREENBERG (1966);
Leukemia suppression in mice by a normal cell metabolite 2-oxopropanal.
In Proc. Pacific Slope Biochemistry Conf. Eugene University or Oregon. p.53.

(13) APPLE, M.A. & D.M. GREENBER (1967);
Inhibition of cancer growth in mice by a normal metabolite.
Life Sci. 6: 2157.

(14) APPLE, M.A. & D.M. GREENBERG (1967);
Arrest of cancer in mice by therapy with normal metabolites. I. 2-oxo-propanal (NSC-79019).
Can. Chemotherapy Rep. 51: 455.

(15) APPLE, M.A. & D.M. GREENBERG (1967);
Normal metabolites which arrest growth of a variety of cancers in mice: oxypropanals.
Proc. Amer. Ass. Can. Res. 8: 3.

(16) APPLE, M.A. & D.M. GREENBERG (1968);
Arrest of cancer in mice by therapy with normal metabolites. II. Indefinite survivors among mice treated with mixture of 2-oxo-propanal and 2,3-dihydroxypropanal.
Can. Chemotherapy Rep. 52: 687.

(17) ARNESEN, K. & J. DEDICHEN (1960);
The influence of a water soluble fraction prepared from the liver on the life span and development of leukemia in AKA mice.
Acta. Pathol. Microbiol. Scand. 50: 347.

(18) ARTH, G.E. (1953);
Reduction of lactones to hydroxyaldehydes with Lithium Aluminum Hydride.
J. Am. Chem. Soc. 75: 2413.

(19) ASHWOOD-SMITH, M.J., D.M. ROBINSON, & J.H. BARNES (1967);
Radiosensitization of bacterial and mammalian cells by substituted glyoxals.
Nature 216: 137.

(20) AUSTRIAN, R. (1952);
Observations on the transformation of pneumococcus in vivo.
Bull. Johns Hopkins Hospital 91: 189.

(21) AVERY, O.Y., C.M. MACLEOD & M. McCARTY (1944);
Studies on the chemical nature of the substance inducing transformation of pneumococcus types.
J. Exp. Med. 79: 137.

(22) BAHNER, C.T., L.R. BARCLAY, G. BIGGERSTAFF, D.E. BILANCIO, G.W. BLANC, M. CLOSE, M.M. ISENBERG & E. PACE (1953);
Compounds for cancer studies.
J. Am. Chem. Soc. 75: 21.

(23) BAKER, N. & L.G. EGYUD (1968);
3-deoxy-D-glucosulose in fed and fasted mouse livers.
Biochim. Biophys. Acta 165: 293.

(24) BANKS, T., C. VAUGHN & L.M. MARSHALL (1955);
Spectrophotometric determination of glyoxal bis-(2,4-dinitrophenylhydrazone) a derivative of glycolaldehyde.
Anal. Chem. 27: 1348.

(25) BARDOS, T.J. (1962);
Reports to the Cancer Chemotherapy National Service Center, National Institute of Health Bethesda, Maryland.

(26) BARDOS, T.J. H.L. GORDON, Z.F. CHMIELEWICZ, R.L. KUTZ & M.V. NADKARNI (1968);
A systematic investigation of the presence of growth inhibitory substances in animal tissues.
Cancer Research 28: 1620.

(27) BARKSDALE, W.L. & A.M. PAPPENHEIMER (1954);
Phage host relationships in nontoxigenic and toxigenic diptheria bacilli.
J. Bacteriol. 67: 220.

(28) BARKSDALE, W.L. (1959);
Lysogenic conversions.
Bact. Rev. 23: 202.

(29) BARKULIS, S.S. (1960);
Biochemical properties of a virulent and an avirulent strain of Group A Hemolytic Streptococcus.
Ann. N.y. Acad. Sci. 88: 0034.

(30) BARNARD, D., L. BATEMAN, A.J. HARDING & H.P. KOCH (1950);
An infrared spetroscopic investigation of double bond structure in simple acyclic terpenes and derivatives thereof.
J. Chem. Soc. p. 915.

(31) BARRETT, F.F. & J.I. CASEY (1970);
Bacteriophage types and antibiotic susceptibility of Staphylococcus aureus.
Arch. Intern. Med. 125: 867

(32) BAUER, E.F. KHALETZKAYA, A. LIVISOVA & L.M. SHABAD (1936);
The inhibition of tumor growth by some fractions of extracts of spleen tissue.
Acta Cancrol. 2: 17.

(33) BAYLOR, M.B. & L.G. EGYUD (1967);
The effect of methyl glyoxal on phage infection of E. coli.
Virology 31: 380.

(34) BAYNE-JONES, S., R.G. HARRISON, C.C. LITTLE, J. HORTHROP & J.B. MURPHY (1938);
Fundamental Cancer Research. Report of a Committee Appointed by the Surgeon General.
Public Health Reports 53: 2121.

(35) BEACHAM, H.H. & M.F. DULL (1951);
Some observations on the browning reaction
Food Res. 16: 439.

(36) BECKER, Y. (1971);
A theory on MRNA species in nerve cells and their rold in brain functions and drug action.
Perspectives in Biol. & Med. 14: 659.

(37) BELKIN, M. & W.G. HARDY (1961);
Relation between water permeability and integrity of sulfhydryl groups in malignant and normal cells.
J. Biophys. Biochem. Cytol. 9: 733.

(38) BELLAMY, L.J. (1960);
The Infrared Spectra of Complex Organic Molecules.
Wiley & Sons, New York.

(39) BERNHEIMER, A.W. (1953);
Synthesis of Type III pneumococcal polysaccharide by suspensions of resting cells.
J. Expt. Med. 97: 591.

(40) BIOLOGY OF CANCER (1957);
Report on the Discussion in Section D (Zoology).
Nature 180: 830.

(41) BIRKINSHAW, J.H., S.E. MICHAEL, A. BRACKEN & H. RAINSTRICK (1943);
Patulin in the common cold. Collaborative research on a derivative of Penicillium Patulam Bainier. II. Biochemistry and Chemistry.
The Lancet II: 625.

(42) BLAIR, J.E. & R.E.O. WILLIAMS (1961);
Phage typing of staphylococci.
W. H. O. Bull. 24: 771.

(43) BLOCH, H. (1950);
Studies on the virulence of tubercle bacilli. Isolation and biologic al properties of a constituent of virulent organisms.
J. Expt. Med. 91: 197.

(44) BLOOM, G.I.M. & L.E. SUTTON (1941);
Electric Polarization and molecular Vibrations: The ethylene dihalides and diacetal
J. Chem. Soc. p. 727.

(45) BLOUT, E.R., V. EAGER & D. SILVERMAN (1946);
Absorption Spectra III. Cyclic B-diketones
J. Am. Chem. Soc. 68: 566.

(46) BOLDINGH, W.H. & E.B. LAURENCE (1968);
Extraction, purification and preliminary characterization of the epidermal Chalone: a tissue specific mitotic inhibitor obtained from vertebrate skin
Europ. J. Biochem. 5: 191.

(47) BOND, C.R., E.C. KNIGHT & T.K. WALKER (1937);
Deoxysones from hexosones by extracts of Aspergilli parasiticus
Biochem. J. 31: 1033.

(48) BONSIGNORE, A., G. LEONCINI, A. SIRI & D. RICCI (1970);
The Lysine catalysed reaction transforming glyceraldehyde into methyl glyoxal
Ital. J. Biochemistry XIX (4): 284.

(49) BOOTH, B.A., D.G. JOHNS, J.R. BERTINO & A.C. SARTORELLI (1968);
Sites of inhibition of DNA synthesis by Kethoxal bis thiosemicarbazone
Nature 217: 250.

(50) BORELLA, L., L.J. JOURNEY & R. HIRAMOTO (1966);
In vivo and In vitro effects of the L-Asparaginase Fraction of guinea pig serum on the 6C3HED Ascites lymphosarcoma
Cancer Res. 26: 2451.

(51) BORREL, A. (1907)
Le probleme du Cancer
Bull. Inst. Pasteur 5: 641.

(52) BOYSE, E.A., L.J. OLD, H.A. CAMPBELL & L.T. MASHBURN (1967)
Suppression of murine leukemias by L-Asparaginase
J. Exptl. Med. 125: 17.

(53) BRAUDE, F.A. (1945);
Organic Chemistry 3. Ultra-violet light absorption and the structure of organic compounds.
Annual Reports Prog. Chem. 42: 105.

(54) BRAUDE, F.A. (1964);
Determination of Organic Structures
in Physical Methods, Pergamon Press, Macmillan Co., New York.

(55) BRAUDE, F.A. & E.R.H. JONES (1945);
Studies in light absorptions Pt. II 2:4 - dinitrophenylhydrazones
J. Chem. Soc. p. 498.

(56) BRAUN, W. (1956);
Cellular products affecting virulence
Ann. N.Y. Acad. Sci. 66: 348.

(57) BRAUN, W. & J. WHALLON (1954);
The effect of DNA and enzyme-treated DNA on bacterial population changes
Proc. Nat. Acad. Sci. 40: 162.

(58) BROWN, W.G. (1951);
in R. ADAMS (Ed.) Organic Reactions p. 469 Vol. VI
John Wiley & Sons Inc., N.Y.

(59) BRUES, A.M., Y. SUBBAROW, E.B. JACKSON & J.C. AUB (1940)
Growth inhibition by substances in liver
J. Exp. Med. 71: 423.

(60) BULLEN, J.J., L.C. LEIGH & H.J. ROGERS (1968);
The effect of iron on the virulence of E. coli for guinea pigs
Immunology 15: 581.

(61) BULLOUGH, W.S. (1965);
Mitotic Functional Homeostasis: A Speculative Review
Cancer Res. 25: 1684.

(62) BULLOUGH, W.S. (1969);
The Chalones
Science J. 5: 71.

(63) BULLOUGH, W.S. (1971);
The actions of Chalones
Agents and Actions 2: 1 (1971).

(64) BULLOUGH, W.S., O.H. IVERSEN & K. ELGO (1967);
The vertebrate epidermal Chalone
Nature 214: 578

(65) BULLOUGH, W.S. & E.S. LAURENCE (1964);
Mitotic control by internal secretion. The role of the Chalone-adrenaline complex
Expt. Cell Res. 33: 176.

(66) BULLOUGH, W.S. & E.B. LAURENCE (1968);
Control of Mitosis in rabbit Vx2 epidermal tumors by means of the melanocyte Chalone.

(67) BULLOUGH, W.S. & E.B. LAURENCE (1968);
Control of Mitosis in mouse and hamster melanomata by means of the melanocyte Chalone.

(68) BULLOUGH, W.S. & E.B. LAURENCE (1968);
Chalones and cancers. Melanocyte Chalone and mitotic control in melanomata
Nature 220: 137.

(69) BULLOUGH, W.S. & T. RYTOMA (1965);
Mitotic homeostasis
Nature 205: 573.

(70) BUNGELER, W. (1930);
Tierexperimentelle and zellphysiologische Untersuchungen zur Frage der allgemeinen Geschwulstdisposition
Frankfurt Ztsch. f. Path. 39: 314.

(71) BUNGELER, W. (1932)
Die Bedeutung der Milz fur das Wachstrum and den Stoffwechsel maligner Tumoren
Frankfurt Ztsch. f. Path. 43: 409.

(72) BURKL, W., I. KRAMER & E. SCHAUENSTEIN (1967);
Über die Bindung α,β-ungesättigter Aldehyde in Zellen and Geweben
A. Naturforschg. 226: 763.

(73) BURNS, J. & D. HOLTMAN (1960);
Biochemical properties of virulent and avirulent Staphylococci
Amm. N.Y. Acad. Sci. 88: 1021.

(74) CALABI, O. (1970);
In vitro interaction of Shigella flexneri with leukocytes and HeLa cells.
J. Inf. Dis. 122: 1.

(75) CANTAROW, A. & B. SCHEPARTZ (1962);
Biochemistry, W.B. Saunders & Co., Philadelphia

(76) COLLINS, F.M. (1964);
The effect of the growth rate on the composition of S. enteritidis cell walls.
Aust. J. Expt. Biol. Med. Sci. 42: 255.

(77) CROSS, A.D. (1960);
An Introduction to Practical Infrared Spectroscopy
Butterworths Inc.

(78) DAMESHEK, W. (1951);
Some Speculations on the myeloproliferative Syndromes
Blood 6: 372.

(79) DAVIS, J.E. (1937);
Biochemical differences between mice of tumor and non-tumor strains and tumor and non-tumor-bearing mice of tumor strains
Canad. Med. Ass. J. 36: 27.

(80) DEDICHEN, J. & P. LALAND (1949);
Anti pernicious anemia factor and white cell count
The Lancet 13: 282.

(81) DOMAGK, E., R. BEHNISCH, F. MIETZSCH & H. SCHMIDT (1946)
Über eine neue gegen Tuberkelbazillen in vitro wirksame Verbindungsklasse
Naturwissenschaften 33: 315.

(82) DOMAGK, G. & C. HACHMAN (1935);
Ein Beitrag zu den bei Transplantattumoren beobachteten Immunostats-resp. Resistenzerscheinungen
Ztsch. f. Krebsforsch. 42: 192.
Am. J. Cancer 25: 825.

(83) DORFMAN, L. (1953);
Ultraviolet-Absorption of Steroids
in Chemical Reviews Vol. 53.

(84) DRUCKREY, H. & D. SCHMAHL (1959);
Tumor destroying properties of normal tissues.
Acta Unio Internat. Contra Cancrum 15: 875.

(85) DRUCKREY, H., D. SCHMAHL & M. RAJEWSKY (1958)
Inactivierung von Krebzellen durch Homogenate normaler Organe bei Ratten
Naturwissenschaften 45 (1): 16.

(86) DUBOS, R. J. (1945);
In The Bacterial Cell p. 200
Harvard University Press, Cambridge, Mass.

(87) DUBOS, R. J. (1954);
In Biochemical Determinants of Microbial Disease
Harvard University Press, Cambridge, Mass.

(88) DUNCAN, I.B.P. & R. COMTOIS (1966);
Hospital Infection by a group of recently recognized Strains of Staphylococcus sureus
Can.Med. Ass. J. 94: 879.

(89) DU PONT, H.L., S.B. FORMAL, R.B. HORNICK, M.J. SNYDER, J.P. LIBONATI, D.G. SHEAHAN, E.H. LA BREC & J.P. KALAS (1971);
Pathogenesis of E. coli. Diarrhea
New Eng. J. Med. 285: 1.

(90) DYER, J.R. (1965);
Applications of Absorption Spectroscopy of Organic Compounds
Prentice-Hall Inc., Englewood Cliffs, New Jersey

(91) EGYUD, L.G. (1965);
Studies on Autobiotics: Retine in human urine
Biochem. J. 96: 19c.

(92) EGYUD, L.G. (1965);
Studies on Autobiotics: Chemical Nature of Retine
Proc. Nat. Acad. Sci. 54: 200.

(93) EGYUD, L.G. (1967);
Studies on Cell Division: the effect of aldehydes, ketones, and a-ketoaldehydes on the proliferation of E. coli
Currents in Mod. Biol. 1: 14.

(94) EGYUD, L.G. (1968);
Studies on Cell Division: the antagonism of a-ketoaldehydes and thiols
Currents in Mod. Biol. 2: 128.

(95) EGYUD, L.G., J.A. McLAUGHLIN & A. SZENT-GYÖRGYI (1966);
Ketone aldehydes in tissues
Proc. Nat. Acad. Sci. 57: 1422.

(96) EGYUD, L.G. & A SZENT-GYÖRGYI (1966);
Cell Division, -SH, ketoaldehydes and cancer
Proc. Nat. Acad. Sci. 55: 388.

(97) EGYUD, L.G. & A. SZENT-GYÖRGYI (1966);
On the regulation of Cell Division
Proc. Nat. Acad. Sci. 56: 203.

(98) EHRLICH, P. (1908);
Referat über die Genese des Carcinoms
Verhandl. d. deutsch. path. Gesellsch. 12: 13.

(99) ELLIOT, W.A. (1960);
Methyl glyoxal formation from aminoacetone by ox plasma
Nature 185: 467.

(100) ELLNER, P.C., C.J. STOESSEL, E. DRAKESFORD & F. VASI (1966);
A new culture Medium for Medical Bacteriology
Amer. J. Clin. Pathol. 45: 502.

(101) ESTERBAUER, H. & W. WEGER (1967);
Über die Wirkungen von Aldehyden auf gesunde und maligne Zellen.
2. Mitt: Synthese von homologen 4-hydroxy - 2-alkenalen I.
Monatsch. Chem. Bd. 98: 1994.

(102) ESTERBAUER, H. & W. WEGER (1967);
Über die Wirkungen von Aldehyden auf gesunde und maligne Zellen. 3. Mitt: Synthese von homologen -4 -hydroxy -2 -alkenalen II.
Monatsch. Chem. Bd. 98: 1884.

(103) EWING, W.H. (1963);
Isolation and identification of E. Coli serotypes associated with diarrheal diseases
U.S. Dept. Health, Eduation, & Welfare. Public Health Service

(104) FARDON, J.C. (1933);
The immunizing properties of various tissues and tissue fractions to the transplantable mouse adenocarcinoma
Bull. Basic. Sci. Res. 5: 5.

(105) FEDEROFF, S. (1960);
Comparison of effect of cerebrospinal fluid and of blood serum on Tissue Cultures
J. Lab. Clin. Med. 56: 431.

(106) FEDEROFF, S. & J. DOERR (1962);
Effect of human blood serum on tissue cultures. III A natural cytotoxic system in human blood serum.
J. Nat. Cancer Inst. 29: 331.

(107) FEDEROFF, S. & S.J. WEBB (1962);
Natural cytotoxic antibodies in human blood sera which react with mammalian cells and bacteria
Nature 193: 80.

(108) FEINGOLD, D.S. (1969);
Biology and pathogenicity of microbial spheroplasts and L-forms
New England Journal of Med. 281: 1159.

(109) FICHERA, G. (1934);
Die biologische Krebsbehandlung und ihre Beziehung zu der Strahlentherapie der Geschwülste
Strahlentherapie 50: 302 (1934).

(110) FIRSHEIN, W. (1960);
DNA synthesis, repiration and virulence in pneumococci
Ann. N.Y. Acad. Sci. 88: 1055.

(111) FIRSHEIN, W. & W. BRAUN (1958);
On the nature of the selective effects of DNA digests on pneumococci of different virulence
Proc. Nat. Acad. Sci. 44: 918.

(112) FIRSHEIN, W. & W. BRAUN (1960);
Effects of enzymatic DNA digests on population changes and DNA synthesis of pneumococci

(113) FODOR, G; J.-P. SACHETTO, A. SZENT-GYÖRGYI & L.G. EGYUD (1967);
Ketone Aldehydes in animal tissues
Proc. Nat. Acad. Sci. 57: 1644.

(114) FOLEY, G.E. & B.P. DROLET (1964);
Loss of neoplastic properties in vitro. I. Observations with S-180 cell lines.
Cancer Res. 24: 1461.

(115) FRANKEL, S. & E. FURER (1915);
Active and Passive immunization with tumor extracts
Wien. klin. Wchnschr. 28: 1433.
Chem. Abs. 10: 2109 (1916).

(116) FREEDLANDER, B. & F.A. FRENCH (1958);
Carcinostatic action of polycarbonyl compounds and their derivatives: II. glyoxal-bis (guanylhydrazone) and derivatives
Cancer Res. 18: 360.

(117) FREEDLANDER, B. & F.A. FRENCH (1958);
Carcinostatic action of polycarbonyl compounds and their derivatives: III. Hydroxymethyl glyoxal-bis (guanylhydrazone)
Cancer Res. 18: 1286.

(118) FRENCH, F.A. & B. FREEDLANDER (1958);
Carcinostatic action of polycarbonyl compounds and their derivatives: 3-ethoxy-2-ketobutyraldehyde and related compounds
Cancer Res. 18: 172.

(119) FRENCH, F.A. & B. FREEDLANDER (1958);
Carcinostatic action of polycarbonyl compounds and their derivatives: IV. Glyoxal bis (thiosemicarbazone) and derivatives
Cancer Res. 18: 1290.

(120) FRENCH, F.A., B.D. FREEDLANDER, A. HOSKING & J. FRENCH (1960);
Carcinostatic Activity of some dicarbonyl compounds and their dihydrazones
Acta Unio Intern. Contra Cancrum 16: 614.

(121) FRIEDMAN, F., L. BURTON & R. KASSEL (1962);
The extraction and refinement of two anti-tumor substances
Trans. N.Y. Acad. Sci. 25: 29.

(122) FURST, A., J. MILLER, H.G. GROSS & W. CUTTING (1960);
A new carcinostatic agent for the Ehrlich ascites tumor
Acta Unio Intern. Contra Cancrum 16: 614.

(123) GABEL, N.W. (1968);
Abiotic formation of phosphoric anhydridge bonds in dilute aqueous conditions.
Nature 218: 354.

(124) GERLICH, N. (1958);
Proc. of 6th Congress of the European Soc. Haematology
Copenhagen 1957. Karger, Basel 2: 276.

(125) GIERKE, E. (1908);
Die hämmorrhagischen Mäusetumoren mit Untersuchungen uber
Geschwülstresistung und Disposition bei Mausen
Beitr. Z. path. Anat. u.z. allg. Path. 43: 328.

(126) GREEN, M.L.
The enzymatic formation of aminoacetone from threonine and
its further metabolism
Biochem. J. 92: 537.

(127) GREGG, C.T. (1968);
Inhibition of mammalian cell division by glyoxals
Expt. Cell Res. 50: 65.

(128) GRIFFITHS, F. (1928);
The significance of the pneumococcal types
Jour. Hygiene 27: 113.

(129) GRIGNARD, V., G. DUPONT & R. LOCQUIN (1950);
Traite de Chimie Organique Vol. VII

(130) GUIDOTTI, G.G., A FONNESU & E. CIARANFI (1964);
Inhibition of amino acid incorporation into the protein of
Yoshida ascites hepatoma cells by glyceraldehyde
Cancer Res. 24: 900.

(130)a GURGO, C. (1971);
Inhibitors of the RNA & DNA dependant polymerase activities
of RNA tumor viruses
Nature (New Biol.) 229: 111.

(131) HACMANN, J.P. (1967);
The reactions of guanosine and other nucleic acid derivatives
with glyoxal, ninhydrin, and related compounds. The preparation
of a novel biacetyl self condensation product.

(132) HAMRE, D., J. BERNSTEIN & R. DONOVICK (1950);
Activity of p-amino benzaldehyde, 3-thiosemi-carbazone on
Vaccinia Virus in chick embryo and in the mouse
Proc. Soc. Expt. Biol. Med. 73: 275.

(133) HARROW, B. & A. MAZUR (1962);
Textbook of Biochemistry 8th Ed. W.B. Saunders Co., Philadelphia

(134) HARMANN, E. (1959);
Investigations of a factor in certain normal tissues
inhibiting ascites tumor growth in a rat and mouse
Brit. J. Cancer. 13: 693.

(135) HEGYELI, A., J.A. McLAUGHLIN & A. SZENT-GYORGYI (1963);
On the chemistry of the Thymus gland
Proc. Nat. Acad. Sci. 49: 230.

(136) HEGYELI, A., J.A. McLAUCHLIN & A. SZENT-GYORGYI (1963);
Preparation of Retine from human urine
Science 142: 1571.

(137) HELLSTROM, N., M. AAMISEPP & G. FUCHS (1964);
On unsaturated lactones and mercaptans. I. ∝-Angelica lactone and thioglycolic acid
Lantbrukshögskolans Annaler 30: 601.

(138) HELLSTROM, N., M. AAMISEPP & G. FUCHS (1966);
On unsaturated lactones and mercaptans. II. β-Angelica lactone and thioglycolic acid
Lantbrukshögskolans Annaler 32: 427.

(139) HELLSTROM, N., S.O. ALMQUIST, M. AAMISEPP & S. RODMAR (1968);
On unsaturated lactones and mercaptans. III. Lactam of 2-(β-carboxyethyl)-2-methyl thiazolidine-4-carboxylic acid from ∝-Angelica lactone (pent-3-en-4-olide) and cysteine, and its NMR Spectrum
J. Chem. Soc. (C) p. 392.

(140) HENDERSON, I. (Personal Communication)

(141) HERBUT, P.A. & W.H. KRAEMER (1960);
Presence of a tumor inhibiting principle in livers
Am. J. Pathol. 36: 105.

(142) HERTWIG, O. & H. POLL (1907);
Zur Biologie der Mäusetumoren
Abh. d. k. preuss. Akad. d. Wissensch. (Supp) 1: 1.

(143) HIGUCHI, S. (1912);
The immunizing power of the placenta, blood, embryonic skin, mammary gland and spleen of different species against carcinoma of the mouse.
in E.F. Bashford (Ed.) Fifth Scientific Report on the investigation of the Imperial Cancer Research Fund, London, 1912

Chem. Abst. 7: 642 (1913)

(144) HIRAMOTO, R., C. TATE & M. HAMLIN (1966);
Guinea pig serum inhibitory factor and asparaginase activity
Proc. Soc. Expt. Biol. Med. 121: 597.

(145) HITCHINGS, G.H. & E.A. FALCO (1946);
Effect of nutrition on susceptibility of mice to pneumococcal infection
Proc. Soc. Expt. Biol. Med. 61: 54.

(146) HODGE, J.E., B.E. FISHER & E.C. NELSON (1963);
Dicarbonyls, reductones, and heterocyclics produced by reactions of reducing sugars and secondary amine salts. A Review.
Am. Soc. Brewing Chemists. 84: 1-41.

(147) HOEKSEMA, H., A.D. ARGOUDELIS & P.F. WILEY (1962);
Chemistry of Actinospectacin. II. The Structure of Actinospectacin
J. Am. Chem. Soc. 84: 3212.

(148) HOLMAN, R.J., W.O. LUNDBERG & G.O. BURR (1945);
Spectrophotometric studies of the oxidation of fats. V. The colour of oxidized fats in Alcoholic alkali
J. Am. Chem. Soc. 67: 1669.

(149) HORI, M. & T. UKITA (1962);
Inhibition in vitro of growth of rat ascites tumor cells by bovine liver extract
J. Biochem. (Tokyo) 51: 322.

(150) ITAMI, S. (1919);
An investigation of the power of mesodermal derivatives to immunized mice against transplantable tumor
J. Cancer Res. 4: 23.

(151) JACKSON, S. & T.W. BURROWS (1956);
The virulence enhancing effect of iron on non-pigment mutants of virulent strains of Pasturella pestis.
Bnt. J. Expt. Pathol. 37: 577.

(152) JACOBS, W.A., A. HOFFMAN & E.L. GUSTUS (1926);
The association of the double bond with the lactone group in the cardiac glycosides
J. Biol. Chem. 70: 1.

(153) JELLUM, E. (1966);
Thiol Content of calf thymus histone fractions
Biochim. Biophys. Acta 115: 95.

(154) JELLUM, E. (1968);
Metabolism of the ketoaldehyde 2-keto-3-deoxyglucose
Biochim. Biophys. Acta 165: 357.

(155) JERZYKOWSKI, T., W. MATUSZEWSKI, N. OTSZONSEK & R. WINTER (1970);
Antineoplastic action of methyl glyoxal
Neoplasma. 17: 1.

(156) JOHNSON, G.D. (1953);
Correlation of color and constitution. I. 2,4-dinitrophenyl-hydrazones
J. Am. Chem. Soc. 75: 272.

(157) JONES, L.A., J.C. HOLMES & R.B. SELIGMAN (1956);
Spectrophotometric studies of some 2,4-dinitrophenyl-hydrazones
Anal. Chem. 28: 191.

(158) KATES, J.R. & J. BEESON (1970);
Ribonucleic acid synthesis in Vaccinia Virus II. Synthesis of Polyriboadenylic acid
J. Mol. Biol. 50: 19.

(159) KEENEY, J. (1957);
Regeneration of carbonyls from 2,4-dinitrophenylhydrazones with levulinic acid
Anal. Chem. 29: 1489.

(160) KELLOG, D.S., W.L. PEACOCK, W.E. DEACON, L. BROWN & C.I. PIRKLE (1963);
Neisseria gonorrhoeae I. Virulence genetically linked to clonal variation.
J. Bacteriol. 85: 1274.

(161) KELLOG, D.S., I.R. COHEN, L.C. NORINS, A.L. SCHROETER & G. REISING (1968)
Neisseria gonorrhoeae II Colonial variation and pathogenicity during 35 months in vitro.
J. Bact. 96: 596.

(162) KELLOG, D.S. & J.D. THAYER (1969);
Virulence of gonococci
Annual Review of Medicine 20: 323.

(163) KENNY, C.P. (In Preparation; Personal Communication)

(164) KENNY, C.P., F.E. ASHTON, B.B. DIENA & L. GREENBERG (1967);
A chemically defined protein-free liquid medium for the cultivation of some species of Neisseria
Bull. World Health Org. 37: 569.

(165) KENNY, C.P. & H. LEES (1965);
The growth of animal cells in bacteriological media
Can. J. Biochem. 43: 933.

(166) KENNY, C.P. & B.G. SPARKES (1968);
Inhibitor of bacterial growth released by human cells in culture
Science 161: 1344.

(167) KEPINOW, L. (1920);
De la résistance au cancer des souris préparées par injection répétées de tissu cancéreux chauffé.
Reunion Soc. Belge. Biol. p. 77.
Chem. Abst. 15: 3319.

(168) KIDD, J.G. & J.E. TODD (1954);
Inhibitory effects of normal guinea pig serum and immune rabbit serum in transplanted A K R lymphomas
Proc. Soc. Expt. Biol. 86: 781.

(169) KIESSLING, K.H. (1963);
Aldehydes as inhibitors of mitochondrial respirations. 1. Effect of biological aliphatic aldehydes on brain and liver mitochondria in vitro
Acta Chem. Scand. 17: 2113.

(170) KISCH, B. (1932);
Beeinflussung der Gewebsatmung durch Methylglyoxal
Biochem. Z . 253: 373.

(171) KLAINER, A.S. & W.R. BEISEL (1969);
Opportunistic Infections: A Review
Am. J. Med. Sci. 258: 431.

(172) KLAMERTH, O.L. (1968);
Influence of glyoxal on cell function
Biochem. Biophys. Acta 155: 271.

(173) KNOCK, F.E., R.M. GALT & Y.T. OESTER (1967);
Protein-sulfhydryl groups in cellular control mechanisms and cancer
J. Am. Geriatrics Soc. 15: 882.

(174) KNOX, W.E. (1960);
Glutathione
in The Enzymes
(P.D. Boyer et al. Eds.) 2: 253.

(175) KOCH, J. (1962);
Aromatic constituents in apple and grape juices
Intern. Fruchtsaft Union Ber. Wiss. Tech. Komm. 4: 57.

(176) KOCH, J. & H. SCHILLER (1964);
Apple Aroma
Z. Lebensm. Untersuch-Forsch. 125: 364.
Chem. Abst. 62: 1509e (1965).

(177) KOENIGSFELD, H. (1914);
Über Versuche zur Immunisierung gegen Mäusekrebs.
Centralbl. f. Bacteriol. (Abt. 1) 73: 316.
Chem. Abstr. 8: 1987.

(178) KREMZNER, L.T. & D.H. HARTER (1970);
Antiviral activity of oxidized polyamines and aldehydes
Biochemical Pharmacology 19: 2541.

(179) KUCHLER, C., W. KUCHLER & W. SCHULZE (1968);
Antiviral effect of Methyl glyoxal-bis (guanylhydrazone)
Acta Virologica 12: 441.

(180) KUN, E. (1950);
Inhibition of succinic dehydrogenase by Methyl glyoxal
J. Biol. Chem. 187: 289.

(181) KVAMME, E. (1958);
Glucose inhibition of respiration in ascites tumor cells
I. PO_4 metabolism in relation to glycolysis and the Crabtree effect.
II. Effect of iodoacetic acid and 2,4-DNP.
III. Relation to the tricarboxylic acid cycle.
IV. Pasteur effect, malonate, and Crabtree effect.
Acta Physiol. Scand. 42: 204.

(182) KWAK, K.S., H.M. KURTZ, E. JAMESON & B. BRADLEY (1963);
Cell culture studies on the tumor inhibiting activity of guinea pig serum
Proc. Assoc. Cancer Res. 4: 36.

(183) KWILAAKSO, E. & T. RYTOMAA (1971);
Erythrocytic Chalone, a tissue specific inhibitor of cell proliferation in the erythron
Cell Tiss. Kinet. 4: 1.

(184) LANDY, M., J.G. MICHAEL, R.J. TRAPPANI, B. ACHINSTEIN, M.W. WOODS & M.J. SHEAR (1960);
An antibody complement system in normal serum lethal to mouse tumor cells
Cancer Res. 20: 1279.

(185) LANKFORD, C.E., C.E. EKLAND & S.Y. ALAMI (1955);
Loss of virulence detected in the homologous serum-soft agar system
Bacteriol. Proc: 88.

(186) LARDY, H.A., V. WIEBELHOUS & K. MANN (1950);
The mechanism by which glyceraldehyde inhibits glycolysis
J. Biol. Chem. 187: 325.

(187) LEEDOM, J.M. & S.S. BARKULIS (1959);
Virulence of streptococci and the quantity of the M protein content of cell walls.
J. Bacteriol. 78: 687.

(188) LEONARD, C.G., R.D. HOUSEWRIGHT & C.B. THORNE (1958);
Effects of some metallic ions on glutamyl polypeptide synthesis by B. subtilis
J. Bacteriol 76: 499.

(189) LEVIN, I. (1909);
Resistance to growth of cancer induced in rats by injection of autolysed rat tissue
Proc. Soc. Expt. Biol. Med. 7: 64.

(190) LEVIN, I. (1910);
Immunity to the growth of cancer induced in rats by treatment with mouse tissue
Proc. Soc. Expt. Biol. Med. 7: 107.

(191) LEWIN, G. (1909);
Die bösartigen Geschwülste.
Leipzig W. Klinkhardt (1909)
Chem. Abstr. 6: 393R.

(192) LEWIS, K.F., E.H. MAJANE & S. WEINHOUSE (1959);
Metabolism of neoplastic tissue: methyl glyoxal formation during glucose catabolism in tumors
Cancer Res. 19: 97.

(193) LEWISOHN, R. (1938);
Effect of subcutaneous injections of concentrated spleen extract on mouse Sarcoma 180.
Surg. Gynec. & Obst. 66: 563.

(194) LI, C.P., W.G. JAINES & N.M. TAURASO (1970);
Studies on inhibition of viral oncognesis
II. Inhibitory effect of L-asparagenase, clam liver extract, and methotrexate on Rous Sarcoma Virus-focus formation
Archiv f.a. gesamte Virusforschung 32: 236.

(195) LI, C.P., O.C. LIU, B. PRESCOTT & E.C. MARTINO (1968);
Antineoplastic activity of clam liver extract
Nature 219: 1163.

(196) LI, C.P., B. PRESCOTT, B. EDDY, G. CALDUS, W.R. GREEN, E.C. MARTINO & A.M. YOUNG (1965);
Antiviral activity of paolins from clams
Ann. N.Y. Acad. Sci. 130: 374.

(197) LI, C.P., B. PRESCOTT, B. EDDY, E.W. CHU & E.C. MARTINO (1968);
Reduced tumor incidence in hamsters inoculated with adenovirus 12 and treated with clam extracts.
J. Nat. Cancer. Inst. 41: 1249.

(198) LIEBERMAN, O. & P. OVE (1960);
Inhibition of growth in cultured mammalian cells by liver abstracts
Biochim. Biophys. Acta 38: 153.

(199) LINVILLE, R.G. & R.C. ELDERFIELDS (1941);
Studies on lactones related to the cardiac aglycones
II Synthesis of β-substituted Δ,α,β-butenolides from ω - acetoxymethyl ketone.
J. Org. Chem. 6: 270.

(200) LITT, M. & V. HANCOCK (1967);
Kethoxal, a potentially useful reagent for the determination of nucleotide sequences in single stranded regions of transfer RNA
Biochemistry 6: 1848.

(201) LORENZUTTI, G., U. SAPIO & E. ESPOSITO (1964);
Studio comparativo di alcuni derivati chetoaldehidici in rapporto alla loro azione inattivante in vitro sul virus influenzale A-PR8.
Boll. Soc. Ital. Biol. Sper. XL: 1221.

(202) MACLEOD, C.M. (1965);
The pneumococci
in Bacterial and mycotic infections of man p. 391
(Eds. R.J. DUBOS & J.G. HIRSCH)
Philadelphia; J.P. Lippencott Co.

(203) MAGANINI, H., M. BECKER, R. MANASSA & G. HASS (1960);
Inhibition of cell growth in vitro by ultrafiltrates of chick embryo extract
Fed. Proc. 19: 386.

(204) MANN, T. (1954);
The biochemistry of Semen and of the Male reproductive tract.
John Wiley & Sons Inc., New York

(205) MARKUZE, Z. (1963);
Effects of Trace Metals on the browning of glucose-lysine solutions
Rocz. Pantsw. Zakladu Hig. 14: 65
Chem. Abstr. 59: 4981a.

(206) MARMASSE, C. (1966);
Chemical nature of free Retine an anti tumor agent
Nature 209: 1346.

(207) MARSHAK, D. & A.C. WALKER (1945);
Effect of liver fractions on Mitosis in regenerating liver
Am. J. Physiol. 143: 226.

(208) MASON, S.F. (1959);
The electronic spectra of N-heteroaromatic systems.
Part I. the $n \rightarrow \pi$ transitions of monocyclic azines
J. Chem. Soc. p. 1240.

(209) MASON, S.F. (1959);
The electronic spectra of N-heteroaromatic systems
Part II. Substituted monocyclic azines
J. Chem. Soc. p. 1247.

(210) MASSARANI, E., D. NARDI, L. DEGEN & M.J. MAGISTRETTI (1966);
Antiviral compounds
XIII. Aminoacethydrazones of aromatic α-ketoaldehydes
J. Med. Chem. 9: 617.

(211) MAZIA, D. (1954);
In Glutathione (S. COLOWICK et al., Eds.)
p. 209 Acad. Press,New York

(212) McARDLE, A.H. (Personal Communication)
McARDLE, A.H., D. MAVRIAS & G. BOUNOUS (1968);
Energy production in the dog intestine following hemorrhagic shock
Surg. Forum. 19: 8.

(213) McCABE, W.R. (1964);
Studies on Staphlyococcal infections
I. Virulence of Staphylococci and characteristics of infections in embryonated eggs
J. Clin. Invest. 43: 2146.

(214) McCABE, W.R. (1966);
Studies on Staphylococcal infections
II. The role of bacteriphage type, antibiotic resistance, coagulase, and other exotoxins in infections in embryonated eggs
Yale J. Biol. Med. 38: 323.

(215) McLIMANS, W.F., G.E. UNDERWOOD, E.A. SLATER, E.V. DAVIS & R.A. SIEM (1957);
Antiviral activity of dicarbonyls and related compounds in embryonated eggs
J. Immunol. 78: 104.

(216) MEADEARIS, D.S. (1969);
Biology and pathogenicity of microbial spheroplasts and L-forms
New Eng. J. Med. 281: 1159.

(217) MELOAN, C.E. (1903);
Elementary Infrared Spectroscopy
Macmillan Co., New York

(218) MENDEL, B. (1929)
Krebzelle und Glycerinaldehyde
Klin. Wschr. 8: 169.

(219) MEYNELL, G. (1961);
Phenotypic variation and bacterial infection.
Symp. Soc. Gen. Microbiol. 11: 174.

(220) MILLER, F.R., P.A. HERBUT & H.W. JONES (1947);
Treatment of lymphoblastic leukemia with crude myelokentric acid
Blood 2: 15.

(221) MILLER, F.R. & D.L. TURNER (1943);
The action of specific stimulators on the hematopoietic system
Amer. J. Med. Sci. 206: 146.

(222) MOHR, U., J. ALTHOFF, V. KINZEL, R. SUSS & M. VOLM (1968);
Chalones and cancers. Melanoma regression induced by Chalone.
Nature 220: 138.

(223) MORTON, R.A. (1965);
Ultraviolet Absorption Spectra
in Biochemistry of Quinones (R.A. MORTON, Ed.)
Acad. Press, London.

(224) MULLIGAN, L.P. & R.L. BALDWIN (1967);
The conversion of acetoacetate to pyruvaldehyde
J. Biol. Chem. 242: 1095.

(225) NAKAYA, K., O. TAKENATA, H. HORINISHI & K. SHIBATA (1968);
Reactions of glyoxal with nucleic acids, nucleotides, and their component bases
Biochim. Biophys. Acta 161: 23.

(226) NAVASHIN, S.M. & I.P. FOMINA (1962);
Inhibition by high molecular weight polypeptide isolated from several bovine tissues
Antibiotiki: 7: 594.

(227) NEEDHAM, J. & H. LEHMAN (1937);
Non enzymatic formation of methylglyoxal from glyceraldehyde
Biochem. J. 31: 1913.

(228) NEEDHAM, D.M., L. SIMINOVITCH & S. RAPKINE (1951);
On the mechanism of inhibition of glycolysis by glyceraldehyde
Biochem. J. 49: 113.

(229) NEUBERG, C. & E. STRAUSS (1945);
Microdetermination of dicarbonyl compounds on the coloring of nitro compounds in alkaline solution
Arch. Biochem. 7: 211.

(230) NOLLER, N. (1965);
Textbook of Organic Chemistry
W.B. Saunders, Philadelphia

(231) OELKERS, W. (1965);
Einbauhemmung C^{14} markierter Nucleoside in DNS and RNS von Ascites Tumorzellen durch D-und L-Glyceraldehyde
Z. Naturforsch (B) 20: 227.

(232) OSER, E.G. & E.E. PRIBRAM (1914);
Über die bedeutung der Milz in dem an malignem Tumor erkrankten Organismus and die Beeinflussung von Tumoren durch Milzbrei
Z. f. exp. Path. Therap. 12: 295.
Chem. Abstr. 8: 166.

(233) OSGOOD, E.E. (1957);
A unifying concept of the etiology of the leukemias, lymphomas and cancers
J. Nat. Cancer Inst. 18: 155.

(234) OTSUKA, H. & L.G. EGYUD (1967);
Nucleic acid and protein synthesis of malignant ascites cells in the presence of liver extract and methylglyoxal
Cancer Res. 27: 1498.

(235) OTSUKA, H. & L.G. EGYUD (1968);
On the formation of 2-keto-3-deoxy D glucose
Biochim. Biophys. Acta 165: 172.

(236) PARSHLEY, M.S. (1961);
Isolation of a growth inhibitor from adult tissue
Excerpta Med. 15: 589.

(237) PARSHLEY, M.S. (1965);
Effect of inhibitors from adult connective tissue on growth of a series of human tumors in vitro
Cancer Res. 25: 387.

(238) PARSHLEY, M.S., C.E. BLACKWOOD & I. MANDL (1965);
Specific tumor inhibitors from normal tissue
Proc. Am. Ass. Cancer Res. 6: 50.

(239) PARSHLEY, M.S. & I. MANDL (1965);
Inhibition of growth of malignant cells in vitro by a component of normal adult connective tissue
Nature 208: 800.

(240) PASCHKIS, K.E. (1958);
Growth promoting factors in tissues
A Review.
Cancer Res. 18: 981.

(241) PAUL, R. (1948);
Bull. Soc. Chim. France, p. 197.

(242) PERKOW, W. (1962);
Constitution and action of biologically active compounds. The function of unsaturated groups. (1962)
Arzneimittel Forsch. 12: 151.
Chem. Abstr. 58: (1963).

(243) PERKOW, W. (1962);
Constitution and effect of biologically active compounds XV. Acid amides, carbamates, thiocarbamates and urea derivatives
Arzneimittel Forsch. 12: 1185.
Chem. Abstr. 58: 11842 (1963).

(244) PETERING, H.G., H.H. BUSKIRK & G.E. UNDERWOOD (1964);
The anti-tumor activity of 2-keto-3-ethoxy butyraldehyde bis (thiosemicarbazone) and related compounds
Cancer Res. 24: 367.

(245) POKIDOVA, N.W., I.I. SMERTENKO & T.P. BUZINA (1965);
Study of the effect of purified preparations of basic polypeptides from animal tissue on tumor cell cultures
Antibiotiki: 10: 137.

(246) POWERS, J., W.P. MURPHY & C. VAN DOOREN (1933);
Leukocytosis following intramuscular injection of liver extract
J. Clin. Invest. 12: 713.

(247) PRIBELA, A. (1966);
Chemical composition of the aromatic substance concentrates
Prumysl Potravin 17: 423.
Chem. Abst. 65: 15146e.

(248) PROCACCINI, R.L., D.R. DeFANTI & J.J. De FEO (1971);
Inhibition of in vitro histone acetylation in a cell free rat uterine system by methylglyoxal and phenylglyoxal
Biochem. Biophys. Res. Comm. 43: 484.

(249) PRYOR, W.A. (1971);
Free radical pathology
Chem. Eng. News June 7, p. 34.

(250) RAO, P.V., S.RAMACHANDRAN & D.G. CORNWELL (1967);
Synthesis of fatty aldehydes and their cyclic acetals (new derivatives for the analysis of plasmalogens)
J. Lipid Res. 8: 380.

(251) RAPHAEL, R.A. (1947);
Compounds related to Penicillinic acid. Pt.II Synthesis of dihydropenicillinic acid
J. Chem. Soc. p. 805.

(251a) RAPHAEL, R.A. (1948);
Compounds related to Penicillinic acid. Pt. III Synthesis of Penicillinic acid
J. Chem. Soc. p. 1508.

(252) RAPKINE, L. (1931);
Ann. Physiol. Physicochim. Biol. 7: 382.

(253) RATNER, S. (1937);
The action of formaldehyde upon cystein
J. Am. Chem. Soc. 59: 200.

(254) RATZENHOFER, M. & J. ZANGGER (1963);
Drastic morphological changes without loss of viability in Ehrlich ascites tumor cells after incubation with strongly diluted formaldehyde

(255) REID, C. (1957);
Excited States in Chemistry and Biology
Butterworths, London.

(256) RENIS, H.E. (1970);
Antiviral Studies with Kethoxal
Ann. N.Y. Acad. Sci. 173: 527.

(257) RIDDLE, V. & F.W. LORENZ (1968);
Nonenzymatic, polyvalent anion catalysed formation of methyl glyoxal as an explanation of its presence in physiological systems
J. Biol. Chem. 243: 2718.

(258) RILEY, H.L., J.F. MORLEY & N.A.C. FREIND (1932);
Selenium dioxide a new oxidizing agent. Part I. Its reactions with aldehydes and ketones
J. Chem. 243: 2718.

(259) RILEY, H.A. & A.R. GRAY (1943);
Phenylglyoxal
Organic Synthesis 2: 509.

(260) ROBERTS, J.D. & C. GREEN (1946);
Absorption spectra of some 2,4-dinitrophenylhydrazones
J. Chem. Soc. 68: 214.

(261) ROGERS, H.J., J.J. BULLEN & G.H. CUSHNIE (1970);
Iron compounds and resistance to infection. Further experiments with Clostridium welchii Type A in vivo and in vitro
Immunology 19: 521.

(262) ROSS, J.H. (1953);
Infrared spectra for analysis of aldehyde and ketone 2,4-dinitrophenylhydrazones
Anal. Chem. 25: 1288.

(263) ROWLEY, D. (1971);
Endotoxins and bacterial virulence
J. Inf. Dis. 123: 317.

(264) RUBIN, M., W.D. PAIST & R.C. ELDERFIELD (1941);
Studies on lactones related to the cardiac Aglycones
I. Synthesis of β substituted Δα,β butenolides from ω-methoxymethyl ketones
J. Org. Chem. 6: 260.

(265) RYTOMAA, T. & K. KIVINIEMI (1968);
Control of granulocyte production I. Chalone and Antichalone, two specific humoral regulators
Cell Tiss. Kinet 1: 329.

(266) RYTOMAA, T. & K. KIVINIEMI (1968);
Control of granulocyte production II. Mode of action of Chalone and Antichalone
Cell Tiss. Kinet 1: 341.

(267) RYTOMAA, T. & K. KIVINIEMI (1968);
Control of DNA duplication in rat chloroleukaemia by means of the granulocytic Chalone
Durop. J. Cancer 4: 595.

(268) RYTOMAA, T. & K. KIVINIEMI (1968);
Control of cell production in rat chloroleukemia by means of the granulocytic Chalone
Nature 220: 136.

(269) RYTOMAA, T. & K. KIVINIEMI (1969);
Chloroma regression induced by means of the granulocytic Chalone
Nature 222: 995.

(270) SANFORD, K.K., R.M. MERWIN, G.L. HOBBS & W.R. EARLE (1959);
Influence of animal passage on a line of tissue culture cells
J. Nat. Cancer Inst. 23: 1061.

(271) SARTORELLI, A.C., A.T. IANNOTTI, B.A. BOOTH, F.H. SCHNEIDER, J.R. BERTINO & D.G. JOHNS (1965);
Complex formation with DNA and inhibition of nucleic acid synthesis by methylglyoxal bis (guanylhydrazone)
Biochim. Biophys. Acta 103: 174.

(272) SATO, T. & D. GROB (1966);
Effect of skeletal muscle extracts on the growth of Ehrlich Ascites tumor cells
Fed. Proc. 25: 292.

(273) SCAIFE, J.F. (1969);
Mitotic inhibition induced in human kidney cells by methyl glyoxal and Kethoxal
Experimentia 25: 178.

(274) SCAIFE, J.F. (1970);
Modification of the cytotoxic action of hydroxypentenal on cultured mammalian cells
Naturwissenschaften 57: 251.

(275) SCHAUENSTEIN, E. (1967);
Autoxidation of polyunsaturated esters in water: Chemical Structure and biological activity of the products.
J. Lipid Res. 8: 417.

(276) SCHAUENSTEIN, E., W. WOHL & I. KRAMER (1968);
Über den Einfluss von Hydroxyenalen (4-hydroxypent-2,3-trans-enal-1) auf DNS und RNS von Ehrlich Ascites Tumorzellen
Z. Naturforschg. 23b: 530.

(277) SCHAUENSTEIN, E., B. WUNSCHMANN & H. ESTERBAUER (1968);
Uber die weitgehend selektive Abtötung subcutan implantierter Ehrlich Ascites Tumorzellen in vivo durch 4-Hydroxyenale, inbesondere 4-Hydroxypentenal
Z. Krebsforschg. 71: 21.

(278) SCHMAHL, D., P. BANNASCH& T.FLASCHENTRÄGER (1958);
Hemmung der cancerolytischen Wirking von Lungen-Homogenaten durch Blut.
Naturwissenschaften 45(11): 270.

(279) SCHOYER, N.H.D. (1959)
The aetiology of leukemias illustrating an alternative concept of the aetiology of malignancy in general
Lancet II: 400.

(280) SCHROEDER, M.P. & E.S. COOK (1939);
The effects of tissue extracts on the respiration and glycolysis of some normal and tumor tissues
Stud. Inst. Divi Thomae 2: 247.

(281) SCHUBERT, M.P. (1935);
Combination of thiol acids with Methyl glyoxal
J. Biol. Chem. 111: 671.

(282) SCHUBERT, M.P. (1936);
Compounds of thiol acids with aldehydes
J. Biol. Chem. 114: 341.

(283) SCOTT, A.I. (1964);
Interpretation of U.V. spectra of natural products
Macmillan & Co., N.Y.

(284) SEEL, F. (1965);
Atomic Structure and Chemical Bonding
Methuen & Co. Ltd., London EC4

(285) SEGAL, W. & H. BLOCH (1957);
Pathogenic and immunogenic differentiation of M. tuberculosis growth in vitro and in vivo
Am. Rev. Tubercul. 75: 495.

(286) SEIFTER, E., E. HENSON & H.D. ISENBERG (1961);
A series of synthetic Antimicrobial Agents I. Chemistry
Antimicrobial Agents Chemotherapy p. 823.

(287) SHAPIRO, R., B.I. COHEN, S-J. SHIUEY & H. MAURER (1969);
On the reaction of guanine with glyoxal, pyruvaldehyde, and Kethoxal, and the structure of acyloguanines. A new synthesis of N -alkylguanines
Biochemistry 8: 238.

(288) SHAPIRO, R. & J. HACHMAN (1966);
The reaction of guanine derivatives with 1, 2-dicarbonyl compounds
Biochemistry 5: 2799.

(289) SHAW, E. (1946);
A synthesis of Protoanemonin, the tautomerism of acetylacrylic acid and of Penicillinic acid
J. Am. Chem. Soc. 68: 2510.

(290) SHEPPARD, N. (1951);
Infrared and Raman frequencies of hydrocarbon groupings.
J. Inst. Petrol. 37: 95.

(291) SIMS, H.S. & N.P. STILLMAN (1937);
Substances affecting adult tissue in vitro. A growth inhibitor in adult tissue.
J. Gen. Physiol 20: 621.

(292) SLATER, T.F. (1969);
Free radical mechanisms in tissue injury
Meth. Achievm. Exp. Path. 4: 30.

(293) SMITH, H. (1968);
Biochemical challenge of microbial pathogenicity.
Bacteriol Reviews. 32: 164.

(294) SMITH, H., A.E. WILLIAMS, R.S. LOWERY & J. KEPPIE (1968);
The derivation of sublines of low and high malignancy from rat and mouse tumours.
Brit. J. Cancer 22: 359.

(295) SPARKES, B.G. & I.G. WALKER (1966);
Acid soluble sulfhydryl compounds in L cells during various conditions of growth.
Can. J. Biochem. 44: 1159.

(296) SPERTI, G.S. (1941);
Radiation, cellular metabolism and cancer resistance.
Stud. Inst. Div. Thomae 3: 17.

(297) STICH, H.F. & M.L. FLORIAN (1958);
The presence of a mitosis inhibitor in the serum and liver of adult rats.
Can. J. Biochem. Physiol. 36: 855.

(298) STEINHOFF, D., T. FLASCHENTRAGER & P. BANNASCH (1958);
Auflösung von Krebzellen durch Homogenate normales Rattenorgane.
Naturwissenschaften 45 (12): 297.

(299) STERNE. M. (1937);
The effects of different carbon dioxide concentration on the growth of virulent anthrax strains.
Ond. J. Vet. Sa. Animal Ind. 9: 49.

(300) SUGIHARA, Y. & F. ARAKI (1966);
Carcinostatic substances prepared from bovine liver.
Ninth. Intern. Cancer Congr. Tokyo. p. 337.

(301) SYLVEN B. & B.O. HOLMBERG (1965);
On the structure and biological effects of a newly discovered cytotoxic polypeptide in tumor fluid.
Europ. J. Cancer 1: 199.

(302) SZENT-GYÖRGYI, A. (1957);
Bioenergetics.
Acad. Press N.Y.

(303) SZENT-GYÖRGYI, A. (1960);
Introduction of Submolecular Biology.
Acad. Press N.Y.

(304) SZENT-GYÖRGYI, A. (1965);
Cell division and Cancer.
Science 149: 34.

(305) SZENT-GYÖRGYI, A. (1967);
On Retine.
Proc. Nat. Acad. Sci. 57: 1642.

(306) SZENT-GYÖRGYI, A. (1967);
Charge transfer and electron mobility.
Proc. Nat. Acad. Sci. 58: 2012.

(307) SZENT-GYÖRGYI, A. (1968);
Bioelectronics.
Science 161: 988.

(307)a SZENT-GYÖRGYI, A. (1968);
Bioelectronics.
Acad. Press N.Y.

(308) SZENT-GYÖRGYI, A., L.G. EGYUD & J.A. McLAUGHLIN (1967);
Ketoaldehydes and cell division.
Science 155: 539.

(309) SZENT-GYÖRGYI, A., A. HEGYELI & J.A. McLAUGHLIN (1962);
Constituents of the thymus gland and their relation to growth, fertility, muscle and cancer.
Proc. Nat. Acad. Sci. 48: 1439.

(310) SZENT-GYÖRGYI, A., A. HEGYELI & J.A. McLAUGHLIN (1963);
Cancer Therapy, a possible new approach.
Science 140: 1391.

(311) SZENT-GYÖRGYI, A., A. HEGYELI & J.A. McLAUGHLIN (1963);
Growth and cellular constituents.
Proc. Nat. Acad. Sci. 49: 879.

(312) SZMANT, H.H. & A. J. PLANINSEK (1950);
The spectra of some 2,4 - dinitrophenylhydrazones.
J. Am. Chem. Soc. 72: 4042

(313) TAKAHASHI, K. (1970);
The structure and function of Ribonuclease T. XI Modification of the single arginine residue in Ribonuclease T. by phenylglyoxal and glyoxal.
J. Biochem. 68: 659.

(314) TARRO, G. & A. FRASCA (1964);
Sull 'azione derivati chetoaldeidici nei riguardi del virus dell encefalomiocardite, e del virus aftoso.
Boll. Della Soc. Ital. di Biol. Sper. XI: 1228.

(315) THOMPSON, R.L., S.A. MINCTON, J.E. OFFICER & G.H. HITCHINGS (1953);
Effect of heterocyclic and other thiosemicarbazones on Vaccinia infection in mouse
J. Immunol. 70: 229.

(316) THORNE, C.B. (1956);
Capsule formation and glutamyl polypeptide synthesis by B. anthracis.
In Bacterial Anatomy, p. 68. E.T.C. Spooner and B.A.D. Stocker,Eds. Cambridge U. Press, England

(317) THORNE, C.B. (1960);
Biochemical properties of virulent and avirulent strains of B. anthracis
Ann. N.Y. Acad. Sci. 58: 1024.

(318) TIFFANY, B.D., J.B. WRIGHT, R.B. MOFFETT, R.V. HEINZELMAN, R.E. STRUBE, B.D. ASPERGREN, E.H. LINCOLN & J.L. WHITE (1957);
Antiviral compounds. I. Aliphatic glyoxals, ∝-hydroxy-aldehydes and related compounds
J. Am. Chem. Soc. 79: 1682.

(319) TIFFANY, B.D., J.B. WRIGHT,R.B. MOFFETT, R.V. HEINZELMAN, R.E. STRUBE, B.D. ASPERGREN, E.H. LINCOLN & J.L. WHITE (1957);
Antiviral Compounds. II. Substituted ∝ -keto-aldehydes and related compounds
J. Am. Chem. Soc. 79: 1687.

(320) TING, W.K., B.K. GUO, Y.H. KAO & C.M. LI (1969);
Retine: Pt. I. Purification and partial characterization of retine from pig liver
J. Chinese Chem. Soc. 16: 164.

(321) TREMBLAY, E.C. (1957);
Proceedings of the 6th Congress of the European Society of Haematology, Copenhagen, Vol. 2, p. 276.

(322) TSAO, M.U., S.T. TEAGAN, P.E. BORONDY & J. HOOG (1965);
Metabolism of dihydroxyacetone in rat liver
Metabolism 14: 246.

(323) UNDERWOOD, G.E. (1968);
Kethoxal treatment of cutaneous Herpes Simplex
Proc. Soc. Expt. Biol. Med. 129: 235.

(324) UNDERWOOD, G.E. & S.D. WEED (1956);
Glyoxal and related compounds as potential blood sterilizing agents
Proc. Soc. Expt. Biol. Med. 93: 421.

(325) UNDERWOOD, G.E. & S.D. WEED (1961);
The effect of carbonyl compounds on Polio virus
Virology 13: 138.

(326) URATA, G. & S. GRANICK (1963);
Biosynthesis of ∝aminoketones and the metabolism of aminoacetone
J. Biol. Chem. 238: 811.

(326a) VERLY, W.G., Y. DESCHAMPS & J. PUSHPATHADAM (1971);
The hepatic chalone. I. Assay method for the hormone and purification of the rabbit liver chalone.
Can. J. Biochem. 49: 1376.

(327) VOGL, O. & W.M.D. BRYANT (1965);
Mechanism of aldehyde polymerization
Rev. Gen. Caoutchouc Plast. Ed. Plast. 2: 224.

(328) VOROBYEV, V. & V.M. BRESSLER (1963);
Unfractionated preparation of histones from normal mammalian tissues as agents inhibiting growth of transplanting tumors
Nature 198: 545.

(329) WAKO, H. (1953);
Report of the peroxidase reaction. CCIXIV Isolation and identification of methylglyoxal (pyruvaldehyde from human milk)
Tohoku J. Exp. Med. 57: 181.

(330) WARBURG, O. (1925);
The metabolism of carcinoma cells
J. Cancer Res. 9: 148.

(331) WASASTJERNA, K., H. TEIR & A. LARMO (1960);
Influence of various tissue extracts and fractions on transplanted myelocytic leukemia in rats
Acta Med. Scan. 168: 467.

(332) WATERHOUSE, D.F., D.A. FORSS & R.H. HACKMAN (1961);
Characteristic odour components of the scent of stink bugs

(333) WATSON, G.F. (1966);
Preliminary report on the effect of a concentrated calf spleen extract on spontaneous mouse tumors
Growth 30: 491.

(334) WENDEL, W.B. (1943);
Respiratory and carbohydrate metabolism of malaria parasites
J. Biol. Chem. 148: 21.

(335) WEHRLI, W. & M. STAEHELIN (1969);
Rifamycin relation of chemical structure and action on RNA polymerase

(336) WERNER, H. (1945);
Growth-promoting and growth-inhibiting factors in rat tumor tissue
Proc. Soc. Exp. Biol. Med. 59: 128.

(337) WHIPPLE, H.E. (1965);
Antiviral Chemotherapy with isatin β-thiosemicarbazone and its derivatives
Ann. N.Y. Acad. Sci. 130: 71.

(338) WHITBY, L. (1951);
Whither clinical pathology?
J. Clin. Path. 4: 129.

(339) WHITE, B. (1938);
The Biology of Pneumococcus
Commonwealth Fund, N.Y.

(340) WHITEHOUSE, M.W. (1967);
Evaluation of potential antirheumatic drugs in vitro using lymphocytes and epithelial cells. The selective action of indoxole, methyl glyoxal and Chloroquinone
J. Pharm. Pharmac. 19: 590.

(341) WILEY, P.F., A.D. ARGOUDELIS & H. HOEKSEMA (1963);
The chemistry of actinospectacin IV. The determination of the structure of actinospectacin
J. Am. Chem. Soc. 85: 2652.

(342) WILLETTS, A.J. & J.M. TURNER (1970);
Threonine metabolism in a strain of Bacillus subtillis. Enzymes acting on methyl glyoxal
Biochim. Biophys. Acta 222: 668.

(343) WISEMAN, B.K., C.A. DOAN & L.A. ERF (1936);
A fundamental reciprocal relationship between myeloid and lumphoid tissue
J. Amer. Med. Ass. 106: 609.

(344) WOOD, S.C. & S.T. CHANG (1945);
The absorption spectrum of methyl glyoxal
Trans. Farad. Soc. 41: 157.

(345) WOOD, F.C. & R.E. PRIGOSEN (1925);
No immunity produced by inoculating irradiated tumor tissue
J. Cancer Res. 9: 287.

(346) YONEDA, M. & A.M. PAPPENHEIMER (1957);
Some effect of iron deficiency on the extracellular products released by toxigenic and non-toxigenic strains of Corynebacterium diptheriae
J. Bact. 74: 256.