Role of Lipids in Formation of Yellow Pigment from p-Aminobenzoic and p-Aminosalicylic Acids. (20365)

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A number of micro-organisms are capable of producing yellow pigment (YP) when grown in the presence of p-aminobenzoic acid (PABA) and p-aminosalicylic acid (PAS) (1-7). The PABA pigment formed by mycobacteria is an oxidation product of PABA of still unknown chemical composition. The conditions under which it is elaborated, especially the influence of environmental changes on the amounts produced, and the sensitivity of the reaction to certain poisons, indicate the importance of oxidative enzymes in its formation. It is surprising therefore that among the many micro-organisms investigated the mycobacteria are by far the strongest YP producers in spite of their slow rate of metabolic activity. This may suggest that the mycobacteria, unlike the micro-organisms of other groups, possess specific mechanisms by which PABA may be oxidized into YP. An important difference existing between the mycobacteria and most other bacteria is the very high lipid content in the first group of organisms which may contain as much as 30% on a dry weight basis. Since the high concentration of lipids, of yet unknown physiological role, is a most distinctive biochemical characteristic of mycobacteria, we have investigated that part played by lipids derived from mycobacteria and other micro-organisms in the formation of YP from PABA and PAS. The ability of animal cells to form a YP from PABA was first described by Kohn and Liversedge(8). Subsequently Bernheim, Bernheim, and Wilbur(9) suggested that the factors responsible for this reaction are oxidized lipids or unsaturated fatty acids, most likely the peroxides or aldehydes and ketones formed during oxidation. When it became apparent that mycobacterial lipids were capable of forming YP from PABA, we extended our investigations to include lipids from various sources such as other bacteria, yeast, the higher plants and animals.

I. Formation of YP by various lipids. Materials. Lipids of Microbial Origin. a) Total lipids from *Mycobacterium smegmatis, Mycobacterium tuberculosis var hominis* (BCG strain) and *Torulopsis lipofera* were prepared by ether extraction of growth mats and centrifugates. b) Purified bacterial lipids were obtained from several sources. Dr. Hubert Bloch kindly supplied us with 6 lipid fractions derived from *Mycobacterium tuberculosis* which had been prepared during the isolation of the cord factor(10). Two esters of bacterial lipids were kindly made available to us by Dr. E. Lederer of Paris, namely methyl phtieonate, an α-β unsaturated acid, and the methyl-ester of coryno-dienic acid, isolated from *Corynebacterium diphtheriae. Lipids of Vegetable and Animal Origin.* Seventeen substances comprising non-drying, semi-drying and drying oils, hydrogenated oils as well as 5 lipids of animal origin (Table II) were tested. *Solvents.* All lipids were dissolved in a neutral organic solvent of low boiling point to yield concentrations of 0.5 to 10%; the solvent used was free of peroxides and similar impurities and did not possess oxidative or reductive properties. The most suitable solvent for most lipids was petroleum ether of C.P. grade. Other suitable solvents were alcohol, acetone and ether. Several of the purified bacterial lipids, insoluble in petroleum ether, were dissolved in ethyl acetate or chloroform. *Substrates.* PABA and PAS are soluble in alcohol or acetone, but insoluble in petroleum ether. When the latter vehicle (suitable for most lipids) was employed, finely pulverized PABA or PAS crystals were suspended in the lipid-solvent solution in a concentration of 10 to 20 mg per ml. For comparative studies it was preferable to employ more homogenous systems consisting of PABA esters (ethyl, butyl and isobutyl-p-amino-benzoates) which are soluble in petroleum ether. However, these esters reacted at a considerably slower rate than free PABA. In several assays PABA or PAS together with certain lipids were dissolved in alcohol or acetone. Although both solvents often contain

TABLE I. Yellow Pigment Formation by Microbial Lipids.

Lipids	Yellow pigment formation*
Total lipids:	
1. *Mycobacterium smegmatis*	+++
2. " *tuberculosis* (BCG strain)	+++
3. *Torulopsis lipofera*	+++
Purified lipids:	
1. Cord factor from *Myco. tuberculosis*	+
2. Non-toxic fractions isolated with cord factor	0
3. Methyl ester of phthienoic acid	+++
4. " " " corynodienic acid	+++

* These plus values represent relative amounts of YP produced by 10 mg PABA or PAS mixed with 5 mg lipid and exposed 4 days to air and filter paper. They correspond to colors produced by the following quantities of butter yellow dissolved in petrol ether and to colorimetric readings (filter 530 mμ).

Dilution	mg/100 ml of solvent	Colorimeter light transm., %	Plus values	Color
1:200	500	18	+++++	Dark yellowish brown
1:400	250	31	++++	Light " "
1:1000	100	45	+++	" " "
1:2000	50	52	++	Amber
1:20000	5	83	+	Yellow
1:200000	.5	96	(+)	Straw yellow
1:2000000	.05	98	(±)	Faint "

oxidative constituents which accelerate the oxidation of PABA or PAS, the color differences between controls in which only PABA or PAS was present and systems which contained the lipids were nevertheless considerable.

Methods. Since only a few milligrams of the pure bacterial lipids and esters were available, a micro-method was devised which permitted the quantitative study of the development of the YP. This new method, similar to the filter paper technique previously described by Mayer, Grimm and Kull(11) for the analysis of water soluble oxidative enzymes, was based upon the following principle: Lipids and substrates (PABA, PABA esters and PAS) were thoroughly mixed and spread on sheets of No. 1 Whatman filter paper. The formation of YP on the filter paper could be quantitatively observed and the chromatic changes compared with color standards prepared by dissolving butter yellow in lard. In performing these tests sheets of the Whatman filter paper (22 x 18 inches) were ruled into 2 inch squares. With a 1 ml pipette, 0.05 or 0.1 ml amounts of the lipid-PABA solutions or suspensions described above were transferred to the center of a square and the solvent evaporated rapidly. The paper was then suspended in a dry atmosphere. The controls for each test consisted of solutions of the lipid material in the solvent, as well as suspensions of solutions of PABA of PAS in the solvent. *Fatty acids and oils* of which larger amounts were available, were tested in the following manner: 500 mg of fatty acid and 100 mg of PABA were dissolved in 3 ml of 1N NaOH, in a 25 ml Erlenmeyer flask, bringing the volume to 5 ml. The solution was then heated to the boiling point and the resulting yellow color compared with color standards. Two ml oil and 100 mg PABA were mixed in a test tube and heated in a boiling water bath until the color remained stable (15 to 20 minutes). Contrary to the filter paper method, this procedure permitted a more rapid estimation of the oxidative power of the lipids.

Results. a) *Controls.* All lipids used were colorless in the amount applied to the paper and no colors developed during several weeks of exposure to air. Areas containing PABA, and especially PAS alone, gradually became brownish but never formed YP. b) *Formation of YP by Bacterial Lipids.* Crude lipid

TABLE II. Yellow Pigment Formation with Various Fatty Acids, Vegetable and Animal Oils, and Fats.

Test substance (5 mg per test)	Pigment formation 5 days	Pigment formation 10 days
Fatty acids		
Oleic	++	+++
Linoleic	++	+++
Stearic	0	0
Palmitic	0	0
Vegetable oils		
Non-drying		
Almond	(+)	++
Castor	0	(+)
Chaulmoogra	0	0–(+)
Olive	(+)	+++
Semi-drying		
Cottonseed	++	+++
Persic	++	+++
Rape	++	+++
Sesame	+++	+++
Drying		
Linseed	+++++	+++++
Poppyseed	+++	+++
Oiticica	+++++	+++++
Safflower	+++	++++
Walnut	+++	++++
Hydrogenated		
Palm	0	0
Cottonseed	0	0
Animal oils		
Sperm oil	0	(+)
Lard "	(+)	+
Lard	+	++
Cod liver oil	++	++++
Ovolecithin	++++	+++++

extracts derived from *Mycobacterium tuberculosis* and *Torulopsis lipofera* were considerably active in transforming PABA and PAS into YP. The two unsaturated acid esters prepared by Lederer from Mycobacterium and *C. diphtheriae* also had strong activity, whereas Bloch's impure cord-factor was only slightly active. The various non-toxic lipid fractions obtained during the isolation of the cord-factor were inactive. The results are shown in Table I. c) *Formation of YP by vegetable lipids.* Most vegetable oils were capable of forming yellow pigment from PABA and PAS, but differed considerably in their pigment forming activity. In general, the activity was directly proportional to the drying properties of the oils. In the group of non-drying oils, only castor and chaulmoogra oils were virtually inactive; almond, olive and palm oils demonstrated weak activities, while peanut oil proved to be a good yellow pigment former. The 4 semi-drying oils were strongly active. The highest activities were found among the drying oils since linseed, oiticica and tung oils formed YP almost immediately upon contact with PABA. Hydrogenated palm and cottonseed oils had completely lost their activities. The results obtained with 5 mg amounts of these vegetable oils mixed with 10 mg PABA or PAS are noted in Table II. d) *Formation of YP by animal Lipids and Fatty Acids.* Animal oils and fats had various degrees of activity. Sperm oil was practically devoid of activity, while lard and lard oil had good activity, and cod liver oil extremely high activity similar to that of drying vegetable oil, oiticica and tung oils. Ovolecithin was found to be very active, producing YP in less than 24 hours. Among the fatty acids, oleic and linoleic acids were powerful oxidizers of PABA and PAS, whereas saturated fatty acids such as stearic and palmitic acids were completely inactive. The results are inscribed in Table II. It has been shown in these experiments that many bacterial, vegetable and animal lipids and certain unsaturated fatty acids are capable of forming YP from PABA and PAS. With the vegetable oils, this activity paralleled the drying properties of the oils. It was therefore desirable to investigate the mechanism involved in this reaction.

II. Assays to determine the active principle in lipids forming YP from PABA and PAS. Wilbur, Bernheim and Shapiro(12) have found that the color reaction of thiobarbituric acid in the presence of oxidized unsaturated fatty acids which parallels the formation of YP from PABA may be produced by peroxides or ketones and aldehydes. However, certain chemical characteristics pointed to aldehydes or ketones rather than to peroxides as the principal agents involved. The transformation of PABA and PAS into YP may be brought about under the following conditions: 1) the presence of double bonds within the lipid or fatty acid molecule, which may act as hydrogen acceptors; 2) the presence and autoxidative formation of peroxides at the ethnoid linkages of the unsaturated fatty acids; 3) the presence of other oxidation products such as aldehydes and ketones, formed either from peroxides or in addition to peroxides. The *oxidative potentials* of these 3 systems are

TABLE III. Influence of "Blowing" (Oxidation) and Heating of Various Lipids and Oleic Acid on YP Formation.

Test substance	Treatment	Ability to form YP after: 48 hr	96 hr	Peroxide No.
a. Microbial lipids				
Myco. smegmatis	0	+	+++	—
	ox*	+++	++++	—
" *tuberculosis*	0	+	++	—
	ox	++	+++	—
Torula lipofera	0	+	++	—
	ox	+++	+++	—
b. Non-drying oils				
Almond	0	0	(+)	49
	ox	(+)	+	303
Castor	0	0	0	10
	ox	0	0	18
Chaulmoogra	0	0	0	22
	ox	(+)?	(+)?	236
Olive	0	+	+	25
	h	++	++	12
	ox	++	++	779
	ox + h	++	++	6
c. Semi-drying oils				
Persic	0	+	++	20
	ox	++	+++	211
Cottonseed	0	+	++	116
	h	++	++	20
	ox	++++	++++	646
	ox + h	+++++	+++++	22
Rape	0	+	+++	20
	ox	++++	++++	120
Sesame	0	+	+++	38
	h	+	+++	17
	ox	+++++	+++++	863
	ox + h	++++	+++++	23
d. Drying oils				
Linseed	0	+	+++++	7
	ox	+++	+++++	101
Oiticica	0	++	+++++	12
	ox	+++++	+++++	160
Poppyseed	0	+	++++	35
	ox	+	++++	694
	ox + h	++++	+++++	0
Walnut	0	+	++++	21
	ox	+++	++++	247
Cod liver	0	++	++++	23
	ox	++++	+++++	74
e. Fatty acid				
Oleic	0	+	++++	40
	ox	+++++	+++++	375

* 0 = untreated; ox = oxidized; h = heated.

different and it is probable that they attack PABA or PAS with different degrees of facility. In order to recognize which factor may actually effect the formation of YP from PABA and PAS, we enhanced the various oxidative principles in a number of various oils. This was achieved by 1) heating at 165°C, 2) *"blowing"*, and 3) *"blowing"* and subsequent heating at 165°C without aeration. Heating at 165°C slightly increased the peroxide content. *"Blowing"*, the technical procedure employed to increase the viscosity of drying oils, consists of heating the oils at 125°C and passing air through the

oils during the heating process for various periods of time. In our experiments, the oils were "blown" with a vigorous stream of air for 24 hours over a steam bath. This procedure considerably increased the viscosity of semi-drying and drying oils, in some instances to an almost resinous consistency. The polymerization of these oils is due to an intense oxidation during the "blowing" process, with an appreciable resultant increase in peroxide content. Subsequent heating of the "blown" oils at 165°C for one and one-half hours reduced the amount of peroxides to the original level with formation of highly reactive aldehydes and ketones. Oils treated by the above procedures were tested for YP activity. Bacterial lipids were oxidized by irradiation with ultraviolet light as indicated by Wilbur, Bernheim and Shapiro(12). As in the preceding experiments, 5 mg of lipid material was mixed with 10 mg PABA or PAS, spread on filter paper and exposed to air.

As seen from Table III, the oxidation of lipids from *Mycobacterium smegmatis, Mycobacterium tuberculosis var. hominis* (H37-Rv) and *Torula lipofera* increases their ability to form YP. Oxidation of non-bacterial lipids has variable effects. In the case of 3 non-drying oils the ability to form YP is not enhanced by oxidation, and only in one instance (olive oil) was it slightly increased. On the other hand, with the semi-drying and drying oils oxidation considerably and regularly enhanced this activity. These results were unexpected since oxidation increased the peroxide content of most oils regardless of whether they belong to the class of drying or non-drying oils. The degree of oxidation as measured by the peroxide numbers was irregular. Only in the case of castor oil did oxidation fail to increase the peroxide content.

Formation of YP and degree of peroxidation did not parallel each other. With the non-drying chaulmoogra oil for instance, the peroxide content increased more than 10-fold, although the ability to form YP remained almost unchanged. In the case of oleic acid the increase in peroxide content was less than 10-fold, but YP formation rose considerably. It was concluded from these observations therefore, that peroxide formation was not the essential factor responsible for the formation of YP by lipids. This hypothesis was confirmed by the results of experiments in which previously oxidized oils, upon heating at 165°C were found to retain considerable YP activity. The oils were heated in order to decrease the peroxide content to the original low level. Consequently, the increased production of YP following the oxidation of certain lipids is apparently due to oxidation products formed from or by the action of peroxides and not by the peroxides themselves. As already pointed out, Wilbur, Bernheim and Shapiro(12) arrived at the same conclusions in the case of thiobarbiturate reaction with unsaturated fatty acids.

Discussion. 1) The present study indicates that many bacterial, vegetable and animal lipids, as well as certain fatty acids, are capable of forming YP from PABA and PAS. Thus three different systems producing these YP are known, namely intact cells of mycobacteria and certain other micro-organisms, phenoloxidase, and lipids. The mechanism by which lipids are capable of forming YP was investigated in order that information might be gained as to the significance of PABA pigment formation and, at the same time, the possible physiological role of bacterial lipids. The results have suggested that the activity of lipids in transforming PABA is not a function of double bonds or of peroxides as such, as first supposed, but is associated with those oxidation products normally produced during rancidification or aging of lipids and fatty acids and which apparently are formed by and from peroxides. Is the formation of YP from aromatic amines by various bacterial lipids an incidental chemical property which has no physiological significance or is it biologically important? It is generally believed that the lipids or waxes of the tubercle bacillus act as a protective sheath against the defense systems of the host. The experiments reported upon in this paper suggest that lipids have a more active and vital role and participate, as with certain animal lipids, in the oxidoreductive cell metabolism as part of the general energy processes. Unsaturated lipids as well as their oxidation products are unstable in air and likely to set up redox systems between non-oxidized and oxidized forms. In this way, they may con-

stitute a hitherto unrecognized oxido-reductive entity in addition to or supplementing the well-known oxido-reductive enzymes of protein nature.

2) The chemical nature of the PABA and PAS pigment formed by lipids is unknown. Earlier studies have suggested that the YP formed from PABA by actively growing mycobacterial cells contains quinoid oxidation products of PABA. Sloane, Fields and Mayer (13) found that highly purified PABA YP had a molecular weight of over 1000000 and contained, in addition to 2 aromatic nitrogenous components, at least 13 different amino acids. It is presumed that the yellow compound formed from PABA by lipids in the absence of proteins has a simpler chemical composition and lends itself to a simpler analysis than the yellow pigments formed in cultures of *M. tuberculosis* or *M. smegmatis*. According to Bernheim, Wilbur and Fitzgerald (14) PABA may condense with aldehydes or ketones to form the YP. On the other hand it is possible that an azo dye is formed from PABA which accounts for the bright and stable color of the YP.

3) The semi-micro filter paper method developed for the study of the YP formed by lipids seems applicable to the elucidation of many oxidative processes. It has the same advantages of application to chemical and enzymatic reactions as the filter paper method developed for the study of the water soluble system of phenoloxidase and other water soluble oxido-reductive reactions(11). Indeed both permit the use of very small amounts of reagents and the observation of many sequential reactions in the course of which colored intermediates or end products are formed, *viz.* melanin and indophenol blue formation, photobiological processes, etc. This method is also useful for the study of reactions during which colorless intermediates or end products are formed which are identified by color reactions such as proteinolysis, carbohydrate metabolism, fat oxidation, etc. A further advantage of this method is the possibility of preventing or interrupting the chemical processes, enzymatic or otherwise, at any stage of development by specific poisons applied during or before color development.

Summary. 1) As in the case of animal lipids and unsaturated fatty acids, various bacterial and vegetable lipids are capable of forming yellow pigment from p-aminobenzoic acid and p-aminosalicylic acid. 2) Oxidation considerably increases the activity of certain lipids to form the pigment. This enhanced activity is apparently not due to an increase in the peroxide content but rather to the formation of other oxidation products such as fatty aldehydes or ketones. 3) These results suggest that bacterial lipids play a role in the PABA pigment formation by growing cultures of mycobacteria. 4) The possible role of lipids in bacterial metabolism as important oxido-redox systems has been discussed. 5) A new filter paper method has been applied for the study of biologic oxidations of lipids and fatty acids.

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